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Biochemical and functional characterization of disease-associated aberrations in Protein Phosphatase 2A

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"Most people say that it is the intellect which makes a great scientist. They are wrong: it is character." ~ Albert Einstein

Bedankt allemaal,

Dorien

LIST OF ABBREVIATIONS

Akt	Protein Kinase B
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANKLE2	Ankyrin Repeat And LEM Domain Containing 2
ANP32a	Acidic nuclear phosphoprotein 32a
APC	Adenomatous polyposis coli
ARF	ADP Ribosylation Factors
ARPP-16/19	cAMP-regulated phosphoprotein
AUL	Acute undifferentiated leukemia
BAF	Barrier-to-autointegration factor
bc	Blast crisis
BSA	Bovine serum albumin
cAMP	cyclic adenosine monophosphate
C/S	Cesarean section
CC	Corpus callosum
CCDC6	Coiled-Coil Domain Containing 6
CCM	Cerebral cavernous mal- formation
Cdk	cyclin-dependent kinase
CGH	Comparative genomic hybridization
CHX	Cycloheximide
CIP2A	Cancerous inhibitor of PP2A
CLL	chronic lymphocytic leukemia
CML	chronic myelogenous leukemia
cMyc	Cellular Myelocytomatosis oncogene
CNV	Copy number variation
CTTNBP2NL	Cortactin Binding Protein 2 N-Terminal Like
COSMIC	Catalogue of Somatic Mutations in Cancer
CT	Computed tomography
DARPP32	Dopamine & cAMP regulated phosphoprotein of 32kDa
DD	Developmental delay
DDD	Deciphering Developmental Disorders
DECIPHER	Database of Chromosomal Imbalance and Phenotype in Humans Using Ensembl Resources
DMEM	Dulbecco's Modified Eagle's medium
DNA	Deoxyribonucleic acid
DTT	DL-Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ENSA	α-Endosulfin
ERα	Estrogen receptor α
ERK	extracellular signal-regulated kinase
FECH	Ferrochelatase
FGFR1OP	Fibroblast growth factor receptor 1-oncogenic partner 2
FIGO	Federation of Gynecology and Obstetrics
FTY720	Fingolimod
GCKIII	Germinal center III kinases
GFP	Green fluorescent protein
GSK3β	Glycogen synthase kinase 3 β
GST	Glutathione S-Transferase
HA	HemAgglutinin
HEAT	Huntington/elongation/A-subunit/TOR
HEC	Human endometrial cancer
HEK293	Human embryonic kidney 293
HNPCC	Hereditary Non-Polyposis Colon Cancer
HR	HEAT repeat
HRP	Horseradish peroxidase
hTERT	Catalytic subunit of telomerase
IB	Immunoblotting
ID	Intellectual disability
IKKε	inhibitor of NF-κB kinase ε
IP	Immunoprecipitation
INHAT	Inhibitor of acetyl transferase
IQ	Intelligence quotient
kDa	Kilodalton
KI	Knockin
KO	Knockout
LATS	Large tumor suppressor kinase
LCMT1	Leucine carboxyl methyltransferase 1
LEM	LAP2-Emerin-MAN1
Leu	Leucine
LRR	Leucine rich repeat
LT	Large T
mAKAP	cAMP-dependent PKA anchoring protein
MAP	Microtubule-Associated Proteins

MAPK	Mitogen-activated protein kinases
MCAP	Megalencephaly-capillary malformation-polymicrogyria
MEK1	Mitogen-activated protein kinase kinase
MIP	Molecular inversion probes
MMR	Mismatch repair
Mob4	Phocein
MPPH	Megalencephaly-polymicrogyria-polydactyly-hydrocephalus
MRI	Magnetic resonance imaging
MS	Mass spectrometry
MSI	Microsatellite instability
MST	Mammalian Sterile 20-like kinase
mRNA	Messenger riconucleic acid
mTOR	Mammalian target of rapamycin
N2A	Neuro-2a
NAP	Nucleosome Assembly Protein
NF-κB	Nuclear factor-κB
NGF	Nerve Growth Factor
NGS	Next-generation sequencing
NLS	Nuclear localization signal
NMDA	N-methyl-D-aspartate receptors
OA	Okadaic acid
P53	Protein 53
PAD	PP2A-activating drug
PBS	Phosphate-buffered saline
PDE	Phosphodiesterase
PDK1	3-phosphoinositide-dependent kinase-1
PEI	Polyethylenimine
PET	Positron emission tomography
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PME1	PP2A Methyl Esterase 1
PMSF	Phenylmethylsulfonyl fluoride
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
PPZ	phenothiazine
PTPA	Phosphatase Two A Phosphatase Activator
PRR14L	Proline rich 14-like
Pwo	Pyrococcus woesei
Rb	Retinoblastoma
REC	Research Ethics Committee
RT-PCR	Reverse Transcription-PCR
SAM	S-adenosylmethionine
Ser	Serine
SET	Suvar 3-9/Enhancer of zeste/Trithorax
SGA	Small for gestational age
SIKE	Suppressor of IKKε
SLMAP	Sarcolemmal membrane–associated protein
SNP	Single nucleotide polymorphism
St	Small t
STK25	Serine/Threonine Kinase 25
STRIPAK	Striatin-interacting phosphatase and kinase complexes
STRIP1	Striatin Interacting Protein 1
SV40	Simian virus 40
TAZ	Tafazzin
TBS	Tris-buffered saline
TEV	Tobacco Etch Virus
TGFβ	Transforming growth factor
TH	Tyrosine hydroxylase
Thr	Threonine
TIPRL	Type-2A interacting protein
TLCK	N-alpha-tosyl-L-lysinyl-chloromethylketone
TrkA	tropomyosin-related kinase A
Tyr	Tyrosine
VEP	Variant Effect Predictor
WAIS	Wechsler Adult Intelligence Scales
WISC	Wechsler Intelligence Scales for Children
WT	Wildtype
YAP1	Yes-associated protein 1

TABLE OF CONTENTS

1 Introduction

INTRODUCTION

1. Protein phosphatases of type 2A

Reversible protein phosphorylation is extremely important in regulating many intra- and inter-cellular processes and is orchestrated by protein kinases and protein phosphatases, which attach and remove a phosphate group to and from a substrate protein, respectively [1, 2]. This post-translational modification can alter the activity, conformation and subcellular localization of target proteins in various signaling pathways, thereby affecting processes like cell proliferation, differentiation, apoptosis, DNA replication, growth and cell division [3]. In a normal cell, the function of one third of the cellular proteins is controlled by phosphorylation [4]. The majority of phosphorylations occurs on Ser/Thr residues with 86.4% on Ser and 11.8% on Thr residues, followed by 1.8% on Tyrosine (Tyr) residues [5]. Paradoxically, 385 Ser/Thr kinases exist in the cell, which are counteracted only by 21 Ser/Thr phosphatases, leading to the misconception that phosphatases are less specific enzymes [4]. Specificity is however achieved through the formation of large protein phosphatase families made up by many multi-subunit holoenzymes and assembled from just a limited number of catalytic subunits. In addition, transient phosphorylations and interactions with regulatory and inhibitory proteins regulate activity and substrate specificity. So, phosphatases are clearly subjected to strict regulation and are equally important as kinases in maintaining proper protein phosphorylation balance [6].

1.1. PP2A: a family of specific serine/threonine phosphatases

Protein Phosphatase type 2A or PP2A represents such a large family of phosphatases and constitutes the majority of Ser/Thr phosphatase activity in the cell together with protein phosphatase 1 (PP1) [7]. PP2A is essential, ubiquitously expressed and highly conserved (from yeast to mammals) [8]. Importantly, its dysfunction has been linked to diverse pathologies, like Alzheimer's disease, diabetes, intellectual disability, cancer, etc. [9-12].

In mammalian cells, PP2A exists as either a dimer (PP2A-AC) or a trimer (PP2A-ACB) [6] (Figure 1). The 36 kDa catalytic subunit (PP2Ac, C) can associate with a 65 kDa structural or scaffold subunit (PR65/A, A) to form the dimeric complex PP2A-AC [13, 14]. For both A and C subunits two isoforms exist in the cell, α and β. Although they possess high sequence similarities, these isoforms are functionally non-redundant [15-18]. Furthermore, Aα accounts for 0.1% of the total cell protein [4]. The core dimeric structure can exist in this form and is estimated to constitute one third of the total PP2A pool [6]. However, the dimer also associates with one of multiple regulatory B-type subunits with a molecular weight ranging from 48 to 130 kDa, to form a trimer. The B subunit is the major regulator of PP2A activity, defining substrate specificity and subcellular localization [19]. The B-type subunits are grouped into four different families, called B55/PR55/B, B56/PR61/B', PR72/B'' and PR93/PR110/B'''. These families are diverse and do not show any sequence similarities [6]. Every family contains at least 4 different isoforms with varying tissue distribution, subcellular localization and developmental expression. Together, the B regulatory subunits comprise a minimum of 26 different transcripts and splice variants encoded by 15 different genes [4]. As a consequence, the combination of the different isoforms of A, B and C can give rise to at least 96 different PP2A holoenzymes, all presumably with different functions in diverse

cellular processes such as transcription, translation, DNA replication, apoptosis and cell division [6].

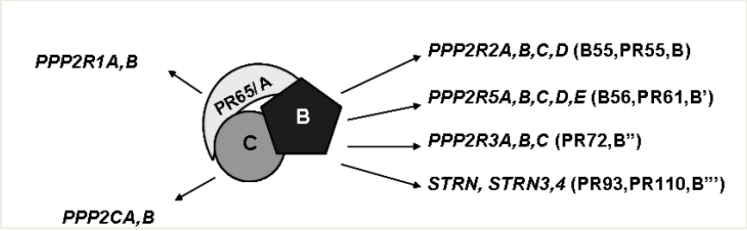

Figure 1: PP2A holoenzyme structure [6]. The PP2A holoenzyme consists of two or three different subunits. The PP2A core dimer (PP2A-AC) consists of a catalytic C subunit that binds with a structural A subunit (PR65/A). This dimer can associate with a regulatory B-type subunit, which determines the activity, substrate specificity and localization of the trimeric complex. There are two isoforms of A and C subunits, namely α and β. The B-type subunits are grouped into four families: B55/PR55/B, B56/PR61/B', PR72 /B'', PR93/PR110/B'''.

The structural A subunit bridges PP2Ac and the regulatory B-type subunit. It consists of a unique structure which contains 15 tandem repeats of 39 amino acids, known as HEAT (Huntington/elongation/A-subunit/TOR) motifs [20]. This 15-HEAT-repeat-molecule adopts a flexible L-shape structure [21]. The catalytic subunit binds with HEAT repeats 11-15, whereas regulatory subunits bind to HEAT repeats 1-8 [22, 23]. The different crystal structures of A in the monomeric (A [21]), dimeric (A-C [14, 24] and A-PR70 [25]) and trimeric (Bα [26], B'γ [20, 27, 28], PR70 [29]) forms reveal that the A subunit undergoes drastic conformational changes upon C and B binding, from a hook shape to a horseshoe-like conformation, probably contributing to the stability of the holoenzymes.

The B55/PR55/B family consists of five isoforms (α, β, β2, γ and δ) encoded by four different genes (*PPP2R2A*, *PPP2R2B*, *PPP2R2C* and *PPP2R2D*). Bα and B δ are widely expressed, while Bβ, Bβ2 and Bγ are highly enriched in the brain. Structurally, the B subunits contain five degenerate WD-40 repeats, i.e. minimally conserved sequences of approximately 40 amino acids that typically end with tryptophan-aspartate, involved in protein-protein interactions [6]. B55/PR55/B subunit-containing complexes regulate apoptosis [30], mitosis [31], transforming growth factor-β (TGF-β) signaling [32], extracellular signal-regulated kinase (ERK) signaling [33], angiogenesis [34], DNA damage signaling [35] and tau dephosphorylation [36].

The B56/PR61/B' subunits represent the largest family (isoforms α to ε) and is encoded by five different genes (*PPP2R5A*, *PPP2R5B*, *PPP2R5C*, *PPP2R5D* and *PPP2R5E*), of which some are alternatively spliced or translated. B'α and B'γ isoforms are abundant in heart, while B'β and B'δ have a high expression in brain. These family members possess a highly conserved central region, which is 80% identical, and very divergent N- and C-terminal domains. It is believed that the central core mediates interactions with A and C, while the divergent ends regulate the substrate specificity and subcellular targeting of the specific B' holoenzyme [6]. Based on amino acid similarity and evolutionary conservation, the B56/PR61/B' family can be divided in two subgroups, B'αβε and B'γδ [37]. These subunits possess a unique feature among the different families because they are phosphoproteins (except B'γ1) [38].

Holoenzymes harboring a B' subunit have other substrates in mitosis/meiosis [31, 39], apoptosis [30], development [40] and dopaminergic signaling in the brain [41]. Importantly, they are also key regulators of several oncogenic targets like Akt, Wnt and c-myc [6, 42-45], and are therefore considered as the main tumor suppressive PP2A complexes.

Human PR72/B" subunits are encoded by three different genes (*PPP2R3A*, *PPP2R3B* and *PPP2R3C*), of which the first two give rise to two alternative splice variants. All are ubiquitously expressed, except B"α2 and B"β2, which are only found in heart and skeletal muscle. The PR72/B" subunits bind and are regulated by Ca^{2+} ions [46]. These PP2A holoenzymes have functions in non-canonical Wnt signaling [33], epidermal growth factor (EGF) signaling [47], DNA synthesis [6, 48], pocket protein dephosphorylation [49] and neuronal signaling [41].

The fourth B-type subunit family consists of the three highly homologous proteins PR110/B"' (striatin), PR93/B"' (S/G2 Nuclear Antigen, SG2NA) and zinedin, encoded by three genes (*STRN*, *STRN3* and *STRN4*). All members are highly expressed in the central and peripheral nervous system, but are also present in many other tissues. They all contain four protein-interaction domains: a Ca^{2+}-calmodulin-binding domain, a caveolin-binding domain, a coiled-coil domain and a WD-repeat domain [50]. Striatin family members serve as scaffold proteins to assemble multiple diverse and large signaling complexes that are involved in numerous functions like e.g. organ size control and development through the Hippo signaling [51], apoptosis [52], cell cycle control [53], Golgi assembly [54], estrogen receptor α (ERα) signaling [55], cell migration [56], and neural development [57]. All striatin family complexes encompass PP2Ac and A subunits. Together with germinal center kinase III (GCKIII) kinases and other components, they form STRIPAK or striatin-interacting phosphatase and kinase complexes [50, 58]. It is believed that PP2A negatively regulates the kinases within this complex. In addition, distinct STRIPAK-like complexes have been discovered that are not yet known to contain both a kinase and PP2A. Interestingly, STRIPAK complexes have been linked to diseases like diabetes, autism, cancer, heart disease and cerebral cavernous malformation [50].

So far, studies addressing the functional roles of different PP2A complexes mainly relied on overexpression and/or depletion of specific B-type subunits in cellular models *in vitro*. Only a limited number of *in vivo* studies have been performed in mammals, showing a role for B56/PR61/B'δ in the central nervous system [59] and for B56/PR61/B'γ in cardiomyocyte maturation and survival [60]. This at least demonstrates that despite significant homology within a given B subunit family, individual B-type subunits serve non-redundant functions in a complex mammalian system.

1.2. PP2A biogenesis: a complex and partially understood process

During the formation or biogenesis of PP2A, the C subunit is synthesized as an inactive enzyme in order to prevent promiscuous/unrestricted activity of free PP2Ac immediately after translation [61]. This is necessary because uncontrolled phosphatase activity would pose a risk to the cell as long as it is not restrained by the interaction with other subunits. As a result, an activation mechanism exists that is coupled to the assembly of the complete

holoenzyme [62]. This biogenesis of active PP2A trimers is a complex step-by-step process that is tightly controlled by several essential PP2A regulators and modulators, including Phosphatase Two A Phosphatase Activator (PTPA), leucine carboxyl methyltransferase 1 (LCMT1), protein phosphatase methylesterase 1 (PME1), Type-2A interacting protein (TIPRL1) and α4.

The currently proposed model for PP2A biogenesis is shown in Figure 2. When PP2Ac is translated as an inactive form, it is believed that α4 binds to it to prevent its degradation [63]. Inactive PP2Ac can then associate with the A subunit, which competes with α4 to form an inactive PP2A-AC that is stabilized by complex formation with PME1. PME1 has a dual role, stabilizing inactive PP2Ac by binding to it and preventing premature PP2Ac methylation via its methylesterase activity [64]. In a next step, PTPA is responsible for the catalytic activation of the complex. PTPA induces a conformational change in PP2Ac, potentially via its peptidyl-prolyl cis/trans isomerase activity on Proline 190 [65], resulting in the dissociation of PME1 [14]. After activation by PTPA, the PP2Ac C-terminal tail is able to be methylated on the free carboxyterminus of the C-terminal Leucine (Leu) 309 by LCMT1 [66]. This modification, on its turn, promotes association with most/certain B-type subunits to form an active holoenzyme [19] (See further). Recently, a role for the A subunit was suggested in facilitating Leu309 methylation by LCMT1 through stabilization of a proper protein fold and an active conformation of PP2Ac, via limiting the space of PP2Ac-tail movement for facilitated entry into the LCMT1 active site, and by conferring weak electrostatic interactions with LCMT1 through the N-terminal HEAT repeats [67]. How TIPRL1 is involved in this process is speculative: it could play a role in the assembly process, or rather later on, in neutralizing PP2Ac upon stress-induced holoenzyme disassembly and, subsequently, making it available for reassembly (as part of the adaptive responses to the stress) or promoting its degradation.

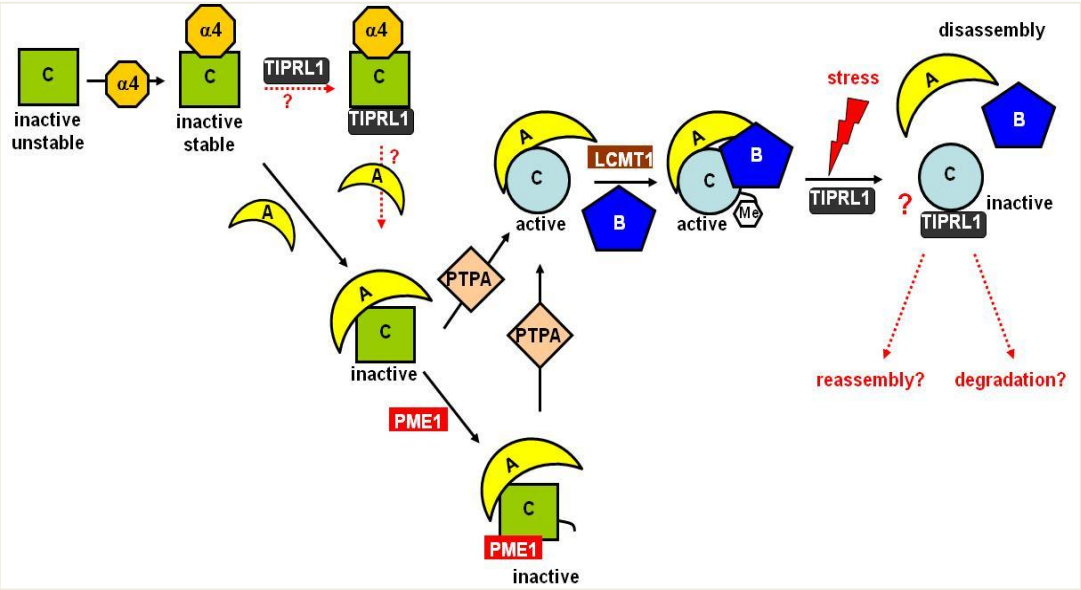

Figure 2: Biogenesis model proposed for PP2A. Figure adapted from [62].

1.3. The role of PP2Ac post-translational modifications in holoenzyme assembly

All PP2A-like phosphatases, including PP4 and PP6, possess a conserved C-terminal tail in their C subunit. Different post-translational modifications (phosphorylation and methylation) of this **304-TPDYFL-309** tail can influence the binding affinity with certain B-type subunits, and therefore, might either play a positive or a negative role in the assembly of a certain holoenzyme. Moreover, fluctuations in the methylation and/or phosphorylation levels of the C subunit tail might provide a theoretical basis for dynamic subunit exchange in PP2A complexes, resulting in altered PP2A targeting and substrate specificity [19]. Indeed, variations in C-tail methylation patterns have been demonstrated in both physiological and pathological circumstances [68].

As introduced in the biogenesis model, a vital post-translational modification of the flexible 304-TPDYFL-309 motif of the C subunit is the **methylation** of the carboxy-terminal Leu309. *In vivo*, 70 to 90% of PP2Ac is estimated to be methylated [19]. It is catalyzed by the S-adenosylmethionine (SAM)-dependent enzyme LCMT1 and reversed by the methylesterase PME1. These PP2Ac modifying enzymes bear indispensable functions since knockout of either LCMT1 [69] or PME1 [70] in mice is lethal. While PME1 is mainly localized in the nucleus, LCMT1 is predominantly present in the cytoplasm and also in the Golgi apparatus and early endosomes. So, spatial control of methylation/demethylation exists [19]. PME1 fulfills a dual role: it reverses the Leu309 methylation on the C-terminal tail of PP2Ac [71] and binds/stabilizes the newly synthesized inactive PP2A in the early steps of PP2A biogenesis [64, 71, 72]. The PP2A-LCMT1 structure reveals that the C-terminal tail occupies the deep active site pocket in the Lid domain of LCMT1. In addition, contacts with the active site of PP2A are necessary to facilitate binding of the C-tail to LCMT1. Like that, conformational changes of the LCMT1 active site are induced to form the C-tail binding pocket and the binding affinity between LCMT1 and PP2Ac is increased. So, a tight link between PP2A methylation and the active conformation of PP2A exist, highlighted by the facts that (1) mutations in the PP2A active site abolish its methylation, (2) two highly potent phosphatase (active site) inhibitors okadaic acid (OA) and microcystin LR prevent PP2A methylation and (3) the C subunit conformation in the PP2A-LCMT1 complex is very similar to that in the PP2A-ACB trimer [73]. According to mutational studies, PP2Ac subunit methylation is essential for binding of the B55/PR55/B subunits but is not absolutely required for interaction with the PR65/A, B56/PR61/B', PR72/B″ and PR93/PR110/B"' subunits (Figure 3). Moreover, B′δ, PR72, PR70 and the striatins [74, 75] can still associate with a deletion mutant of the entire C-tail, suggesting that these B-type subunits do not need stabilizing contacts with the catalytic subunit within the holoenzyme. For B'αβε subunits, Leu309 methylation is not essential but facilitates the incorporation in the holoenzyme. In these cases, a contact with PP2Ac is required [74]. For B'γ, the PP2Ac C-terminal determinants facilitating its incorporation in the holoenzyme have not been reported. Recently, the crystal structure of an A-PR72 PP2A dimer was published, confirming that at least PR72 is able to associate with the A subunit without the need for C subunit binding [25].

The C-terminal tail motif also undergoes **phosphorylation** of Tyr307 and Thr304. Either Tyr or Thr phosphorylation leads to inhibition of PP2A activity [62]. Tyr307 can be phosphorylated by different Tyr kinases, such as pp60v-src, pp56lck, and the epidermal

growth factor and insulin receptors. This Tyr307 phosphorylation is increased by OA-mediated PP2A active site inhibition, implying that there is a reactivation mechanism for PP2A via auto-dephosphorylation [76]. This also implies that PP2A could function as a phospho-tyrosine phosphatase. The formation of B55/PR55/B and B56/PR61/B'-containing PP2A complexes is inhibited by Tyr307 phosphorylation, possibly because addition of a bulky phosphate on Tyr307 prevents access of the tail into the C-tail binding pocket of LCMT1, therefore preventing methylation [77]. On the other hand, only the formation of B55/PR55/B-containing PP2A complexes is inhibited by Thr304 phosphorylation, which occurs during mitosis [78] and does not affect Leu309 methylation (Figure 3).

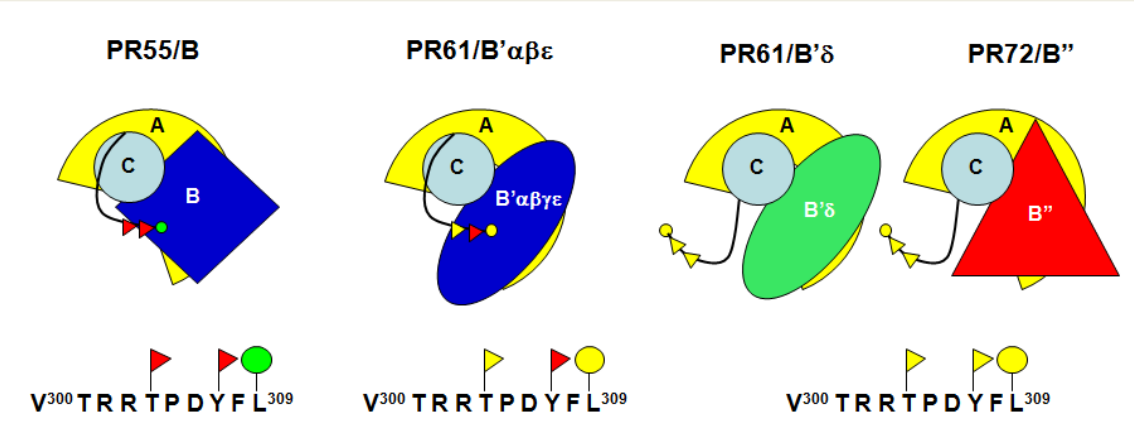

Figure 3: Different PP2A holoenzyme families require specific codes of post-translational modifications on the PP2Ac C-terminal 304-TPDYFL-309 tail for stable integration within trimeric holoenzymes. Flags represent a phosphorylation event and methylation is indicated by circles. Red: not modified, green: modified, yellow: no preference. Figure adapted from [19].

2. PP2A alterations in disease

Alterations in PP2A function and/or activity have been reported in many human diseases, including Alzheimer's disease, diabetes, intellectual disability, cancer, etc. [9-12]. For the puspose of this thesis, I will here discuss in more detail the pathological role of PP2A alterations in human cancer and intellectual disability.

2.1. Cancer

Cancer is a generic term for a group of diseases, characterized by uncontrolled growth, invasion and potentially metastasis of cells. The transformation from normal into malignant cells, called tumorigenesis, is a multistep process in which genetic or epigenetic alterations activate oncogenes and/or inactivate tumor suppressors. When cells become malignant, they acquire essential common characteristics or hallmark capabilities, reviewed by Hanahan and Weinberg [79], which include: sustained chronic proliferation, growth repression evasion, apoptosis evasion, replicative immortality, angiogenesis induction, invasion and metastasis, avoidance of immune destruction, deregulation of cellular energetics, genomic instability and increased mutation rates. These hallmarks are common, but the order in which they are acquired can differ between cancer types and subtypes. Moreover, the role of a specific

genetic aberration may vary substantially between tumors, in the sense that it can partially or fully contribute to the acquisition of a certain hallmark, or that this event may aid in the simultaneous acquisition of several distinct hallmarks. Nevertheless, independently of how and when these hallmarks are acquired, at the biological endpoint of cancer, the same hallmarks will be shared by all types of human tumors.

2.1.1. PP2A, a well-established tumor suppressor

Since the large family of PP2A holoenzymes is involved in nearly every cellular process, it comes of no surprise that malfunctioning of PP2A can contribute to the acquisition of the hallmarks of cancer. PP2A is indeed considered as an important tumor suppressor in the cell. Several observations firmly establish this:

(1) The pharmacological PP2A inhibitor Okadaic Acid (**OA**) is a potent tumor promoter, underscoring a negative role for PP2A in tumorigenesis. OA is a polyether fatty acid produced by marine dinoflagellates and causes diarrhetic shellfish poisoning [80]. In 1998, it was found to bind to PP2Ac, via a hydrophobic cage not found in other Ser/Thr phosphatases, and to potently inhibit its phosphatase activity [3]. Moreover, when injected into mice, OA causes cancerous laesions on the skin and tumors in the liver and stomach [80].

(2) The expression of **viral tumor antigens** by small DNA tumor viruses plays an important role in cell transformation, since they can bind to cellular proteins involved in signaling pathways controlling cell growth, thereby affecting their normal functioning [43]. Moreover, these viral tumor antigens can inactivate tumor suppressors, including p53, retinoblastoma (Rb) and PP2A. For instance, small t antigen of simian virus 40 (SV40 st) or polyoma virus are able to inactivate PP2A by binding to the A subunit and substituting for the B subunit in the holoenzyme [81].

(3) In a cellular model of transformation, the so-called **HEK-TER system** reported by Hahn et al. 1999, inhibition of PP2A by SV40 st is essential to cause the full transformation of human cells immortalized by hTERT, SV40 large T (LT) and oncogenic Ras [82]. Transformation was evaluated *in vitro* by anchorage-independent growth and *in vivo* by tumor formation in immune-deficient mice.
With an increasing amount of cell divisions, the telomeres become increasingly shortened, leading to genomic instability and cell death. Expression of the catalytic subunit of telomerase hTERT ensures a stable length of the telomeres, thereby preserving chromosome integrity and cell viability. SV40 LT inhibits the tumor suppressors p53 and Rb, and expression of oncogenic Ras stimulates cell growth (MAPK pathway), survival (Akt pathway) and migration (Ral pathway). At least two of these Ras effectors need to be activated in order for human cells to become tumorigenic. Also, sole expression of Ras in normal cells causes oncogenic stress-induced senescence, therefore p53 and Rb need to be inactivated to circumvent this and cause cellular transformation [83]. Of note, members of the Ras signaling pathways are the most mutated oncogenes in human tumors [82]. Together, co-expression of hTERT, Ras and SV40 LT results in the immortalization of human cells. To achieve complete transformation, PP2A needs to be inhibited by SV40 st [84]. Indeed, PP2A-binding defective mutants of SV40 st are not able to cause transformation within the HEK-

TER system [37], plus reduction of Cα levels (accompanied by the degradation of multiple PP2A subunits) nearly completely mimics the tumorigenic phenotype caused by SV40 st expression, confirming that PP2A is the major cellular target of SV40 st [84, 85]. By manipulating the levels of A in the HEK-TER system, it was revealed that a 50% reduction of Aα could completely transform cells, while complete deletion causes apoptotic cell death [86]. Interestingly, the main holoenzyme affected by 50% loss of Aα is AB'γC, as opposed to other PP2A complexes which were less affected by Aα suppression [86]. This observation indicates that dynamic subunit exchange could occur *in vivo* due to competition of the B-type subunits for binding to Aα.

The HEK-TER system was also exploited to identify specific PP2A subunits involved in cell transformation by scoring to what extent loss-of-function of a certain subunit could mimic the tumorigenic phenotype caused by SV40 st. Three regulatory B subunits, B'α, B'γ and PR72/PR130, and the biogenesis regulator PTPA were identified as being important for protection against cellular transformation [87]. Several key oncogenic proteins were disturbed in these tumorigenic cells: B'α and PR72/PR130 suppression caused increased expression of c-myc, while suppression of B'γ resulted in Akt activation and increased β-catenin-dependent transcription (canonical Wnt pathway). All three pathways were affected by PTPA suppression, consistent with its role in PP2A biogenesis and holoenzyme formation. Indeed, a decreased level of C-tail methylation was detected together with reduced interaction between A and C [87]. Importantly, while PTPA suppression was able to very closely mimic cell transformation caused by SV40 st expression, suppression of the individual B-type subunits could only partially transform cells. In addition, overexpression of B'γ could only partially rescue the SV40 st tumorigenic phenotype, suggesting that SV40 st likely affects multiple tumor suppressive PP2A complexes [86]. Several additional studies confirm the importance of PP2A's tumor suppressive function on the Wnt, Akt and c-myc pathway. For instance, constitutive PI3K signaling [88], a combination of constitutive active Akt and Rac1 [89], and oncogenic c-myc (T58A mutant, unable to be dephosphorylated on Ser62 by PP2A) [90] replaced SV40 st in anchorage-independent growth assays. However, it is likely that additional oncogenic pathways controlled by PP2A are involved. For instance, loss of the cyclin-dependent kinase (CDK) inhibitor p27/Kip1 also substitutes for SV40 st [91]. Finally, either LCMT1 knockdown or PME1 overexpression cause cell transformation (evaluated via anchorage-independent growth) only in combination with B'γ suppression, resulting in upregulation of Akt and p70S6 kinase pathways [92]. Also, Cancerous Inhibitor of PP2A (CIP2A), a cellular inhibitor of PP2A activity towards oncogenic c-myc, is able to completely mimic the tumorigenic phenotype of SV40 st [93].

(4) Perhaps, the most convincing evidence sustaining a tumor suppressive role of PP2A is the *in vivo* data from two PP2A knockout (**KO**) **mice**, obtained in our laboratory. Both *Ppp2r5d* (encoding PR61/B'δ) and *Ppp2r4* (encoding PTPA) KO mice show indeed spontaneous cancer phenotypes (unpublished work).

2.1.2. Genetic alterations of PP2A in tumor cells

Not surprisingly, a growing amount of PP2A-inactivating mechanisms in cancer is being discovered, further sustaining its important tumor suppressive properties. These include

mutations in PP2A subunit encoding genes, aberrant expression of PP2A subunits and regulatory proteins involved in PP2A biogenesis (PTPA, PME1 and TIPRL1), and overexpression of cellular PP2A inhibitors.

Mutations in and aberrant expression of PP2A subunit encoding genes

In different types of cancer, the A and B-type subunits have been found to be mutated or abnormally expressed.

(1) Reduced expression of Aα was found in 43% of gliomas [94]. In addition, several mutations of the Aα subunit, identified at low frequency (E64D in lung cancer, E64G in breast carcinoma and R418W in melanoma), cause defects in holoenzyme formation through haploinsufficiency [95-97]. In accordance, a knock-in mouse expressing the E64D mutation showed a 50 to 60% increase in the incidence of lung cancer induced by benzopyrene [17]. In the last few years, however, it became apparent that the frequency of Aα mutants is much higher in certain subtypes of endometrial cancer, ranging roughly from 20% to 40% [98-105]. This suggests an important role for Aα (and PP2A) in the pathogenesis of uterine cancer. Many of these mutations (P179L/R, R182W, R183G/Q/W, R249H, S256F/Y, W257C/G, R258C/H/Y) are recurrent and cluster together in HEAT repeats 5 and 7.

(2) Aβ mutations have been discovered in colorectal, lung, breast and gastric cancers and some of these can result in increased activity of RalA, due to complete loss of RalA dephosphorylation, and tumor formation [18]. The Aβ gene locus is prone to loss-of-heterozygosity and subsequent loss of this locus is reported in cervix, ovary, stomach, bladder and breast carcinomas and in melanoma [106].

(3) Mutations in B-type subunits occur at low frequency in a wide variety of cancers. For example, in melanoma (B'γ), c-kit-positive acute myeloid leukemia (Bα and multiple B'), lung cancer (Bα and B'γ) and breast cancer (Bα). These mutations result in defects in paxillin dephosphorylation, double strand DNA repair, p53 functioning and Akt dephosphorylation [35, 107-112]. To further elaborate on this, a search in the cBioportal for Cancer Genomics database (http://cbioportal.org) for aberrations in the different PP2A subunits revealed some additional interesting observations. Firstly, Cβ and Bα are obviously deleted in a large number of cancers, for instance in up to 13.1% of metastatic prostate adenocarcinoma and up to 14.7% of prostate adenocarcinomas, respectively. Another obvious observation is the amplification of B'α and B'δ in up to 12.4% in liver and breast cancer and up to 10% in esophageal carcinoma, respectively. Bβ is mostly amplified (most frequently in kidney renal clear cell carcinoma (16.9%)) and mutated (highest frequency in colorectal adenocarcinoma (5.6%)). PR72/PR130 has the highest frequency of amplification (13.5%) and mutation (9.4%) in lung squamous cell carcinoma. Cα was found overexpressed in kidney renal clear cell carcinoma (16.1%). B'β,γ,ε and PR110/B''' (striatin), PR93/B''' (SG2NA) and zinedin show mixed aberrations, not exceeding frequencies above 8%.

Alterations in regulators of PP2A biogenesis

α4 is found highly expressed in hepatocellular carcinoma (HCC), lung cancer, and breast cancer [113]. PME1 is also overexpressed in several cancers like glioblastoma, endometrial cancer, breast cancer, melanoma, head and neck cancer, etc. with a frequency of 5-10%

(cBioportal). Thus, increased PP2A demethylation may be tumorigenic. Heterozygous loss of *PPP2R4*, the gene encoding PTPA, was discovered at strikingly high frequencies in uterine, lung, liver, renal breast and pancreatic cancer and in melanoma (cBioportal). This loss may be associated with the presence of less active PP2A in the cell, responsible for the tumor formation. Besides heterozygous loss of *PPP2R4*, other alterations, like mutations, were found at a lower frequency in various cancers (cBioportal).

Overexpression of cellular PP2A inhibitors

By far the most frequent PP2A aberration in cancer is the overexpression of cellular inhibitors of PP2A, the most studied being SET (Suvar 3-9/Enhancer of zeste/Trithorax) and CIP2A. This occurs in a variety of cancer types, often with high frequency and associated with cancer aggressiveness and poor outcome. In my review article, 'Cellular inhibitors of Protein Phosphatase PP2A in cancer' (Chapter 3, [114]), the mechanisms of action, regulation and function of these PP2A inhibitors are highlighted, and it is discussed how this knowledge might be exploited for therapeutic use.

Together, the above findings highlight the multiple mechanisms by which PP2A can be inactivated in cancer, underscoring the fact that PP2A is an important tumor suppressor. Specifically for hematological malignancies, these mechanisms causing aberrant PP2A functioning are reviewed in 'The basic biology of PP2A in hematologic cells and malignancies' (Chapter 4 [115]). Although reluctantly, the combined data have increased the interest in exploiting 'PP2A reactivation' as a therapeutic strategy in human cancers, as discussed later on [116]. Nevertheless, the clinical utility of using the "PP2A status" as a stratification marker in human tumors cells still largely remains underestimated and underexploited. This is particularly relevant in cancer types where PP2A aberrations occur with high frequency, such as certain subtypes of endometrial cancer.

2.1.3. Epidemiology of endometrial cancer

Endometrial cancer is the most common gynecological malignant disease; others are ovarian, cervical, vulval and vaginal cancer. Worldwide, endometrial cancer is the sixth most common malignant disorder with approximately 290,000 new cases annually and it is the fourth most common cancer in Belgian women after breast, colorectal and lung cancer ([117], Stichting Tegen Kanker). In 2010, 1450 (5%) new cases and 214 deaths were reported in Belgium. Newly diagnosed patients have a 5-year relative survival rate of 80%, ranging from 94.5% for early disease to 19.5% for advanced disease [118]. Over 90% of cases occur in women older than 50 years of age, with 63 as a median age [119]. The most common symptoms patients suffer from, include abnormal uterine bleeding and vaginal discharge, presenting in 90% of the cases. Patients with advanced disease may also experience abdominal or pelvic pain, early satiety, abdominal distension, or change in bowel or bladder function, symptoms often associated with advanced ovarian cancer [120]. Of all the gynecologic cancers, only cervical cancer can be detected by a standard screening test, called the Papanicolaou or Pap test, and this early in the disease when treatment is most effective. For endometrial cancer, the diagnosis is based on the histology of endometrial tissue obtained during a biopsy with the Pipelle aspiration catheter. To determine the stage of the disease, clinical examination,

transvaginal ultrasound, computed tomography (CT) scan, magnetic resonance imaging (MRI), and integrated positron emission tomography and computed tomography (PET/CT) scan can be performed to evaluate the presence of metastases. Preoperative staging aids in determining the proper treatment options for patients and is based on the international Federation of Gynecology and Obstetrics (FIGO) system. 72% of patients are diagnosed with stage I disease, meaning that the tumor is confined to the body of the uterus. When the tumor has invaded the cervical stroma, but does not extend beyond the uterus, patients suffer from stage II endometrial cancer, as is the case in 12% of patients. Stage III defines local and/or regional spread (with for example vaginal involvement and lymph node metastasis), present in 13% of patients. 3% suffers from stage IV disease, meaning that the tumor invaded the bladder and/or bowel mucosa, with or without the presence of distant metastases, mostly in the lung [117, 121]. Tumors may additionally be grouped according to their level of differentiation in G1 (well differentiated), G2 (moderately differentiated) and G3 (poorly or undifferentiated) [117].

Traditionally, endometrial carcinoma is classified into two main groups, as defined by Bokhman [122]: type I and type II (Table 1).

Type I endometrial carcinoma (87-90% of cases [123]) is composed of low grade endometrioid carcinoma, which are estrogen dependent and develop from endometrial hyperplasia [100]. Most women are diagnosed after menopause and the stage at diagnosis is commonly FIGO stage I and II. Established risk factors are early onset of menstruation, nulliparity, late menopause and unopposed estrogen exposure due to estrogen replacement therapy to control menopausal symptoms, estrogen-producing tumors, tamoxifen treatment, polycystic ovarian syndrome, etc. [120]. In addition, insulin resistance, associated with obesity (causing high estrogen production in adipose tissue) and diabetes, can co-occur. Excessive insulin levels will stimulate insulin growth factor pathway, leading to tumorigenesis [124]. Insulin also inhibits the production of globulin, a sex hormone binding protein, resulting in increased levels of the sex hormones androgen and estrogen. It also promotes synthesis of androgens in the ovaries. An epidemic of obesity in high-income countries is likely the reason for a difference in incidence of endometrial cancer in high-income vs. low-income countries (5.5% vs. 4.2%). However, the specific mortality is higher in the latter, due to a higher prevalence of the aggressive, high-grade cancer type. Other risk factors are age and a family history of Hereditary Non-Polyposis Colon Cancer (HNPCC), also known as Lynch Syndrome, which is an inherited cancer syndrome causing mainly colon cancer, but also other cancer types, most commonly endometrial cancer. Inactivating mutations in DNA mismatch repair (MMR) genes cause this disease. Patients with HNPCC have a 20 to 70% risk for developing endometrial cancer depending on the specific MMR mutation [125]. Interestingly, a reduced risk is associated with the use of combination oral contraceptive pills or progesterone secreting intra-uterine devices. Smoking also reduces the risk for endometrial cancer, especially in postmenopausal women [120]. Type I endometrial cancer is typically diagnosed at early stage before extra-uterine metastasis and, therefore, has an overall favorable prognosis. Surgery is curative in many cases. This type is characterized by high frequency genomic alterations affecting *PIK3CA*, *PIK3R1*, *PTEN*, *KRAS*, *FGFR2*, *ARID1A* (BAF250a), and *CTNNB1* (β-catenin), as well as microsatellite instability (MSI) due to epigenetic silencing of MMR genes [126]. So, mainly the PI3K/Akt/mTOR (cell growth and

survival) and Wnt/β-catenin signaling (gene transcription and development) pathways drive tumorigenesis in these tumors.

Type II endometrial carcinoma includes mainly the uterine serous adenocarcinoma (2.9-10.5% of cases [123]) and the clear-cell adenocarcinoma (2.2-3.2% of cases [123]). Serous adenocarcinoma is estrogen independent and arises in atrophic endometrium and endometrial polyps from preinvasive lesions called serous endometrial intraepithelial carcinoma [100]. Although this type constitutes only a small percentage of all endometrial cancers, it accounts for a disproportionately high number of deaths due to its high aggressiveness and high tendency to metastasize. These tumors are typified as FIGO stage III and IV. Moreover, type II endometrial carcinomas are highly resistant to conventional chemotherapy and recurrence is almost inevitable. These tumors tend to be aneuploid and are characterized by frequent genomic alterations affecting *TP53* (p53), *PPP2R1A* (PP2A Aα), *HER-2/ERBB2*, *PIK3CA*, and *PTEN*; plus dysregulation of E-cadherin, p16 and BAF250a. So, again the PI3K/Akt/mTOR and Wnt/β-catenin signaling seem to be important, together with inactivation of tumor suppressors p53 and PP2A.

Table 1: Traditional classification of endometrial cancer. Table constructed with data obtained from [123, 126, 127]. *PPP2R1A* is highlighted in bold.

	Type I	**Type II**
Features		
Background endometrium	Hyperplasia	Atrophy
Estrogen dependent	Yes	No
Myometrial invasion	Superficial	Deep
Metastatic spread	Low	High
Prognosis	Favorable	Unfavorable
Prototypical histological type	Endometrioid	Serous
Stage at diagnosis	Early (FIGO stage I and II)	Advanced (FIGO stage III and IV)
Common genetic aberrations		
PTEN mutation	52-78%	1-11%
PIK3CA mutation	36-52%	24-42%
PIK3CA amplification	2-14%	46%
PIK3R1 mutation	21-43%	0-12%
KRAS mutation	15-43%	2-8%
AKT mutation	3%	0%
ARID1A mutation	25-48%	6-11%
CTNNB1 mutation	23-24%	0-3%
TP53 mutation	9-12%	60-91%
***PPP2R1A* mutation**	**5-7%**	**15-43%**
FGFR2 mutation	12-16%	1%
HER2 amplification	1%	27-44%
HER2 overexpression	3-10%	32%
Nuclear accumulation of β-catenin	18-47%	0%
E-cadherin loss	5-50%	62-87%
Loss of function of p16	8%	45%
MSI	20-45%	0-5%

Important to emphasize is that an overlap exists between type I and type II endometrial tumors and that heterogeneity is present within each of these types. To clarify, low-grade endometrioid and high-grade serous adenocarcinoma integrate well in the Bokhman model, but 10 to 19% of endometrioid adenocarcinomas are high-grade and have features that are intermediate between type I and II or are more resembling those of type II endometrial

cancer [127]. Likewise, not all serous adenocarcinomas show prototypical type II characteristics. Moreover, none of the mutations in any of the disease genes identified so far can be exclusively assigned to a specific type. For instance, mutations in *TP53* are present in 90% of the serous type and 10% of low-grade and 30% of high-grade endometrioid adenocarcinomas [127]. Consequently, the Cancer Genome Atlas Research Network recently proposed a genomic classification, which can aid in reclassification of the different subtypes of endometrioid and serous carcinomas and the identification of potential targets for targeted treatment of different subgroups of the disease (Table 2). From these data, it is clear that mutations in *PPP2R1A* are associated with poor outcome and aggressive behavior of both the endometrioid and serous types of endometrial adencarcinoma.

Table 2: Features of the four genomic classes of endometrioid and serous carcinomas, as defined by The Cancer Genome Atlas Network. Table adapted from [127]. *PPP2R1A* is highlighted in bold.

	POLE (ultramutated)	MSI (hypermutated)	Copy-number low (endometrioid)	Copy-number high (serous-like)
Copy-number aberrations	low	Low	Low	High
MSI	Mixed (high, low, stable)	High	stable	Stable
Mutation rate	Very high	High	Low	Low
Genetic aberrations	*POLE* (100%) *PTEN* (94%) *PIK3CA* (71%) *PIK3R1* (65%) *FBXW7* (82%) *ARID1A* (76%) *KRAS* (53%) *ARID5B* (47%)	*PTEN* (88%) *RPL22* (37%) *KRAS* (35%) *PIK3CA* (54%) *PIK3R1* (40%) *ARID1A* (37%)	*PTEN* (77%) *CTNNB1* (52%) *PIK3CA* (53%) *PIK3R1* (33%) *ARID1A* (42%)	*TP53* (92%) ***PPP2R1A* (22%)** *PIK3CA* (47%)
Histological type	Endometrioid	Endometrioid	Endometrioid	Serous, endometrioid, and mixed serous and endometrioid
Progression-free survival	Good	Intermediate	Intermediate	Poor

The golden standard treatment of endometrial carcinoma is **surgery**, including total hysterectomy (surgical removal of uterus and cervix) and bilateral salpingo-oophorectomy (surgical removal of fallopian tubes and ovaries). The type and FIGO stage determines the precise surgical procedure and the need for additional lymph node dissection and omentectomy. Moreover, the need for adjuvant therapies is evaluated based on information obtained from surgery [117, 119, 121, 128]. These adjuvant therapies include radiotherapy, chemotherapy, hormone and targeted therapy. Regarding **radiotherapy and chemotherapy**, depending on the FIGO stage and grade of the tumors, pelvic radiotherapy, vaginal brachytherapy and/or chemotherapy are performed, possibly in combination [119]. The combination of the chemotherapeutics carboplatin and paclitaxel is the standard adjuvant therapy for stage III endometrial cancer and is also standard first-line therapy for metastatic or recurrent disease [129]. **Hormonal therapy** is recommended for endometrioid histologies. The main hormone treatment for endometrial cancer employs

progesterone-like drugs called progestins which slow down the growth of cancer cells. Progestins represented the first-line treatment for endometrial cancer in the 1970s and are still used in appropriate settings, particularly for low-grade tumors that recur long after primary therapy. Tamoxifen, which is an anti-estrogen drug often used in breast cancer treatment, may also be used in the treatment of advanced or recurrent disease. Furthermore, gonadotropin-releasing hormone agonists are a way to lower estrogen levels in women who still have functioning ovaries. Aromatase inhibitors can block estrogen production in adipose tissue. These drugs are used in the treatment of breast cancer, but may also be utilized to treat endometrial cancer. The use of antiangiogenic agents, like bevacizumab, is currently under investigation. Recent understandings of the signaling pathways underlying tumorigenesis in endometrial cancer led to the evaluation of **targeted therapies** against these affected signaling molecules. For instance, the PI3K/Akt/mTOR pathway can be targeted at different levels via multiple drugs, for example with Akt, mTORC1, isoform-specific PI3K, Pan-PI3K, dual PI3K/mTOR and mTORC1/2 inhibitors [130]. Currently, however, the benefit of blocking the PI3K/Akt/mTOR pathway is disappointing and limited by cross-talk with the Ras/Raf/MEK pathway (Figure 4). Therefore, dual blockage might be more efficient in the future [131].

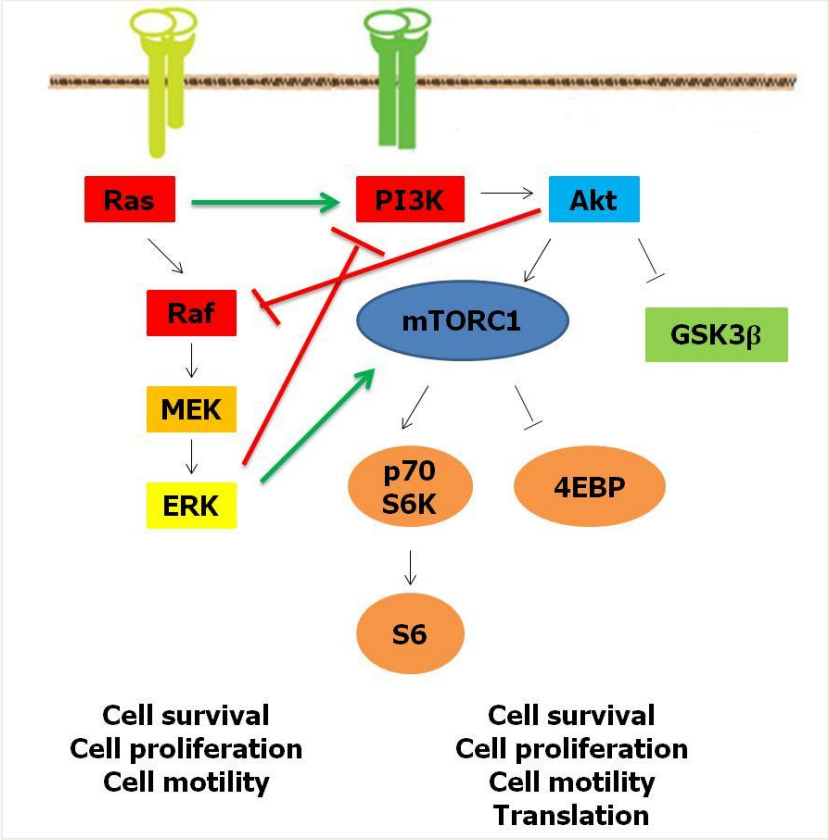

Figure 4: Pathway crosstalk exists between the Ras/Raf/MEK and PI3K/Akt/mTOR pathways. These pathways regulate each other via cross-activation (green) and cross-inhibition (red), often the basis for resistance. Figure adapted from [131].

2.1.4. PP2A aberrations in endometrial cancer

From the previous chapter it is clear that until the molecular pathogenesis of endometrial (serous) carcinoma is better understood, therapeutic interventions to improve the clinical outcomes of these patients remain empirical. The high frequency of mutations in *PPP2R1A,* hinting towards an important role of aberrant PP2A functioning in the pathogenesis, may be one of the opportunities that could be further exploited in this respect.

Historically, a publication from 2010, reporting ***PPP2R1A* mutations** in 7% of ovarian clear-cell carcinoma [132], prompted the evaluation of the *PPP2R1A* mutational status in other gynecological cancers via whole genome sequencing and targeted (exon 5 and 6) approaches [100-103, 105, 133-135]. These studies revealed that somatic mutations in *PPP2R1A* occur at high frequency (18.4-43.2%) in the serous type of endometrial cancer, while the percentage is low (2.5-6.9%) in the endometrioid subtype. Since *PPP2R1A* mutations are specifically correlated with high grade endometrial carcinoma, both serous and endometrioid of origin (genomic classification of The Cancer Genome Atlas Research Network), these findings link the presence of these mutations to aggressiveness of the tumors and poor patient outcome [127]. The heterozygous mutations identified include P179L/R, R182W and R183G/Q/W, located in HEAT-repeat 5, and R249H, S256F/Y, W257C/G and R258H, located in HEAT-repeat 7; they all have been recurrently found in ovarian and in endometrial cancer (Table 3). Because structural and functional studies of PP2A indicate that the mutated HEAT-repeats 5 and 7 directly contact the B subunits, it was hypothesized that the mutations might affect PP2A holoenzyme assembly [22, 23, 96, 97, 136]. The mutations described in endometrioid endometrial carcinoma and in ovarian carcinoma seem to more frequently involve residues R182 and R183, while in the serous endometrial tumors residues S256 and W257 and P179 seem to be much more affected. However, the significance of this uneven distribution is unclear [126]. Another interesting finding is the fact that *PPP2R1A* mutations occur in endometrial serous carcinomas but not (or barely) in ovarian serous carcinomas. This suggests that, although both tumor types share several clinical and pathological features, their pathogenesis is likely different. Therefore, the *PPP2R1A* status could also be used to distinguish these two serous cancer types [100, 101]. Lastly, no correlation was found between the occurrence of mutations in *PPP2R1A* and other genes frequently mutated in serous endometrial cancer, like *TP53*, *KRAS* and *PIK3CA* [103, 133].

Table 3: Overview of the *PPP2R1A* mutations found in endometrial and ovarian carcinoma.

	Endometrial		Ovarian		
	Endometrioid	**Serous**	**Endometrioid**	**Serous**	**Clear-cell**
[132]					7.1% (3/42) R183G (1) R183W (1) R182W (1)
[101]	5% (3/60) R183Q (2) R249H (1)	40.8% (20/49) P179R (6) P179L (3) R182W (1) R183W (2) S256F (6) S256Y (1) W257G (1)	12.2% (5/41) P179R (1) R183W (2) S256Y (1) W257G (1)	0% (0/62)	4.1% (2/49) R183W (1) W257C (1)[§] R258C (1)[§]
[103]	6.7% (2/30) R183W (1) R182W (1)	5/26 (19.2%) S256Y (1) P179R (1) S256F (2) W257G (1)	10% (4/40) R183W (2) R183Q (1) R182W (1)	0% (0/91)	9.1% (4/44) R183W (3) R183Q (1)
[102]	6.9% (19/276) Low-grade R183Q (4) R183W (4) H87Y (2) R249H (1) R48Q (1) S256F (1) P179L (1) S219L (1) Q237R (1) S219L (1) VE99del (1) 182insV (1) 10% (3/30) High-grade R182W (1) R221W (1) R183W (1)	43.2% (16/37) P179R (8) S256F (4) T102K (1) R183W (1) P179L (1) V182M (1)			
[133]	2.5% (3/118) W257C (1) E216K (1) R258Y (1)	32% (8/25) R258H (1) P179R (3) R258Y (1) P179L (2) W257C (1)			
[100]		18.4% (14/76) P179R (7) R183W (3) S256F (1) S256Y (1) W257G (1) W257C (1)			
[105]		20% (6/30) P179R (4) S256F (2)			

Table 3: Overview of the *PPP2R1A* mutations found in endometrial and ovarian carcinoma. (Continued from previous page)

	Endometrial		Ovarian		
	Endometrioid	**Serous**	**Endometrioid**	**Serous**	**Clear-cell**
[134]			6.6% (1/15) High-grade P179R (1)	4.5% (1/22) R183W (1)	4.5% (1/22) R183Q (1)
[135]			2.7% (1/37) W257C (1)	0% (0/76)	0% (0/43)
[137]		25% (13/52) P179R (5) S256F (3) W257S (1) W257C (3) L286P (1)			
[138]	4.5% (14/307) S256F (1) R183W (2) R258C (1) R258H (1) R221W (1) L173M (1) A41T (1) A164V (1) R105Q (1) R28C (2) A114V (1) R144C (1) V298L (1) A136T (1) Q237R (1)	27.9% (12/43) S256F (2) P179R (7) S256Y (1) L_27splice (1) V220M (1)			

Worthwhile to mention is that *PPP2R1A* is also found mutated in endometrial carcinosarcoma. Two studies report frequencies of 26.8% (15/56) [139] and 21.4% (9/42) [102]. The recurrent mutations included P179R, S256F, R183W and S219L. Carcinosarcoma is a rare cancer of the endometrium, with a prevalence of less than 2%. It is a highly aggressive cancer, likely to present in an advanced stage [123]. Furthermore, *PPP2R1A* mutations were detected in 20% (4/20) of patients diagnosed with undifferentiated carcinoma. This relatively uncommon neoplasm is classified as a type of endometrioid carcinoma, although undifferentiated carcinoma displays a more aggressive behavior [99].

Besides *PPP2R1A* mutations, two **mutations in *PPP2R5C***, the gene encoding B'γ, were identified in endometrioid endometrial carcinoma, namely T182M and L168V [102].

PME1 overexpression was recently also detected in the endometrioid adenocarcinoma cell lines, RL95-2, Ishikawa, and ECC-1; and in 83% (24/29) of type I endometrial carcinomas [140]. This overexpression results in decreased PP2A activity, increased cell proliferation and metastases by causing ERK and Akt hyperphosphorylation. Increased PME1 levels also led to increased anchorage-independent growth and tumor formation *in vivo*. In addition, an enhanced interaction with PP4c, a tumor promoter, was found. However, whether PME1 overexpression activates PP4 to promote cell proliferation or inhibits PP4 to counterbalance PP2A inhibition is unclear [140].

2.2. Intellectual disability

Intellectual disability (ID), also called mental retardation, is defined as a collection of diverse and genetically heterogeneous disorders primarily characterized by central nervous system defects of varying severity. It can present as isolated cognitive defects or in association with additional neuromuscular, metabolic, dysmorphic or psychiatric features. The disability originates before the age of 18. Typically, patients have limitation in two areas: intellectual functioning and adaptive behaviors. Intellectual functioning is the ability to learn new and complex information, to learn new skills, to solve problems and make decisions, and is measured by standardized intelligence quotient (IQ) tests like the Wechsler Adult Intelligence Scales (WAIS) and the Wechsler Intelligence Scales for Children (WISC). The average IQ is 100 and ID has been defined as an IQ less than 70, with different severities: mild (70-51), moderate (49-36), severe (35-21) and profound (less than 20). Adaptive behaviors are the conceptional, social, and practical skills necessary to cope in day-to-day life, such as self-direction, interpersonal communication and relationship, personal care, etc. [141, 142]. ID is the leading socio-economic problem of health care in Western countries, exceeding the cost for dementia and even cancer [142]. Management of the disorder often requires a multidisciplinary approach, involving neurology, orthopedics, ophthalmology, audiometrics, cardiology, physiotherapy, psychology and dermatology. The prognosis of the patients depends on the severity of the symptoms. Early deaths have been reported rarely and are mainly due to feeding difficulties, complex cardiac heart disease and arrhythmia [142].

2.2.1. Epidemiology of intellectual disability

ID is thought to affect about 1 to 3% of the population worldwide, with in between 0.3 and 0.5% of the population suffering from a severe form. This prevalence tends to be higher in developing compared to developed countries due to a variety of non-genetic factors like poor health care and malnutrition. In addition, inbreeding is associated with reduced cognitive performance. Other risk factors are low birth weight and prenatal and perinatal complications. Fetal alcohol exposure is the most common preventable cause of ID in the western world. Furthermore, moderate to severe ID is approximately 1.4 times more frequent in males than in females, for which the reason is still unknown. It is believed that mild ID is multifactorial, while severe forms are largely caused by catastrophic genetic defects, including chromosomal aberrations and mutations.

Cytogenetically visible chromosomal aberrations account for almost 15% of all ID cases, including Down syndrome (trisomy 21) which is the most important single cause of ID with a prevalence of 1/750 to 1/800. Nevertheless, microdeletions and microduplications, too small to be detectable by conventional karyotyping, seem to be equally important and were previously overlooked causes of ID. X-linked gene defects are thought to be responsible for 10 to 12% of the ID found in males [142, 143]. However, this percentage is not sufficient to explain higher prevalence of cognitive impairment in males compared to females, meaning there must be additional factors. ***De novo* mutations** represent an increasingly important cause of ID [144]. A *de novo* mutation affects the offspring but is not detectable in the parents. These mutations are typically present in the reproductive cell, sperm or egg, of one

of the parents and once transmitted to the child they are present in all cells and tissues. Postzygotic mosaicism means that the mutation arises during the mitotic cell divisions that generate the embryo, causing only a subset of cells/tissues to harbor the mutation. Regardless of the specific type of mutation, they are inherited relatively rarely because individuals suffering from these disorders are less likely to bear offspring, placing these mutations under strong negative selection. Consequently, the presence of the disorder in fact reveals the ongoing appearance of new *de novo* mutations.

Approximately 80% of *de novo* mutations are paternal in origin, due to 'selfish spermatogonial selection' [145]. Because sperm undergoes many more cell divisions across the reproductive age compared to the female gamete, somatic mutations build up in the sperm which is proven to be associated with an increased transmission of *de novo* mutations onto the offspring of older men. This build-up causes dysregulation of the RAS pathway leading to within-testis proliferation of mutant sperm cells, a process akin to oncogenesis. Whole exome sequencing of parents and offspring showed that the total number of mutations strongly correlates with paternal age in which an increase of approximately two point mutations per year was shown, corresponding to a doubling of mutations every 16.5 years. Indeed, a link was already established between advanced paternal age and other neurological disorders, like autism, bipolar disorder, epilepsy, Alzheimer's disease, etc. [145].

2.2.2. Diagnosis – whole genome and exome sequencing

Unfortunally, the cause of ID is still unknown in up to 60% of cases [146]. It is becoming more and more clear that ID, but also schizophrenia and autism, represent a heterogeneous collection of rare monogenic disorders, of which the large majority is still unknown [147]. Identifying the causative genes is crucial to provide molecular diagnosis for patients, to enable testing for gene carriers and prenatal testing, and to understand the underlying pathology of the disease leading to therapies. Recent advances in next generation sequencing technologies, enabling reliable whole genome sequencing and whole exome sequencing, have drastically changed the process of disease gene identification. Instead of focusing on the whole genome, whole exome sequencing is mainly performed. Exomes represent only 2% of the genome, but roughly 85% of known genetic causes of disease affect these protein coding regions [147]. Moreover, whole genome sequencing generates massive outputs, is time-consuming and is too costly to be used as a routine gene discovery technique. The cost of whole exome sequencing is 2 to 3 times lower compared to whole genome sequencing [148]. Different exome capturing techniques have been developed to enrich exomes before sequencing, like solid-phase and liquid-phase hybridization, polymerase-mediated capture and regional capture [149]. However, this is never complete. As tens of thousands of variants can be identified in each exome, it is important to select the pathogenic variants. For *de novo* mutations, this is achieved through child-parent exome sequencing to identify mutations not present in the parents but present in the child (=*de novo*). The result of such a 'trio-sequencing' approach is a limited number of potential pathogenic variants, since the average exome contains only 0 to 3 *de novo* mutations [147]. Of consideration is the fact that advances in NGS approaches comes with the inevitable identification of mutations unrelated to the condition in question, called secondary or

collateral mutations [150], which obviously requires development and application of consistent ethical guidelines when application of this technique is considered.

In conclusion, whole exome (and genome) sequencing has provided clinicians and researchers with an increasing amount of genes involved in ID, but mechanistic insights and treatments have lagged behind. Therefore, efforts need to be made to overcome this gap.

2.2.3. PP2A aberrations in intellectual disability

Recently, several large-scale screening studies reported *de novo* (sometimes recurrent) mutations in multiple PP2A genes, suggestive of an important role of PP2A dysfunction in ID (Table 4).

Table 4: De novo mutations in PP2A genes discovered in ID patients.

PP2A Gene	*De novo* mutation	
PPP2R1A	P179L (1)	[151]
	R182W (2)	[151]
PPP2R5B	S161L (1)	[152]
PPP2R5C	T157del (1)	[152]
PPP2R5D	P53S (1)	[153]
	E198K (3)	[151, 152]
	E200K (2)	[152]
	P201S (1)	[151]
	W207R (1)	[154]

(1) The Deciphering Developmental Disorders (DDD) study is a large collaboration between 24 Regional Genetics Services throughout the UK and Republic of Ireland, including scientists at the Wellcome Trust Sanger Institute, which aims to identify in large scale new genetic causes for developmental disorders via child-parent whole exome sequencing. The project includes almost 14,000 patients with a developmental disorder. Published data from the first 1133 children revealed *de novo* mutations in ***PPP2R1A***, encoding the Aα scaffold subunit, and in ***PPP2R5D***, encoding B'δ [151]. *PPP2R1A* mutations included P179L and R182W, the latter found in two patients. The *PPP2R5D* mutations identified were P201R and E198K, recurrent in two cases and in one case in another study [151, 152]. Additional unpublished DDD data of 4295 children in total show additional *PPP2R1A* and *PPP2R5D* mutant cases. **Intriguingly, for *PPP2R1A*, the mutations are identical to some of the ones identified in endometrial cancer.**

(2) Other studies also reported sites mutated in *PPP2R5D* (P53S, E200K, P201R and W207R) by child-parent whole exome sequencing [152, 154] and by targeted sequencing of a panel of 21,000 genes [153]. While the P53S mutation localizes in the B'δ-specific N-terminal domain, a region likely involved in substrate binding, all other reported B'δ mutations cluster in a conserved loop directly facing the A and C subunits. Remarkably, the E198 residue makes direct contact with the C subunit and this is the most recurrent mutation among the B'δ cases.

(3) A S161L mutation in *PPP2R5B*, encoding B'β, and a T157del mutation in *PPP2R5C*, encoding B'γ, were identified by trio-based exome sequencing studies in 111 overgrowth

patients and their unaffected parents [152]. Interestingly, these residues map to the same ID-associated conserved loop present in all B56/PR61/B' family members (Figure 5).

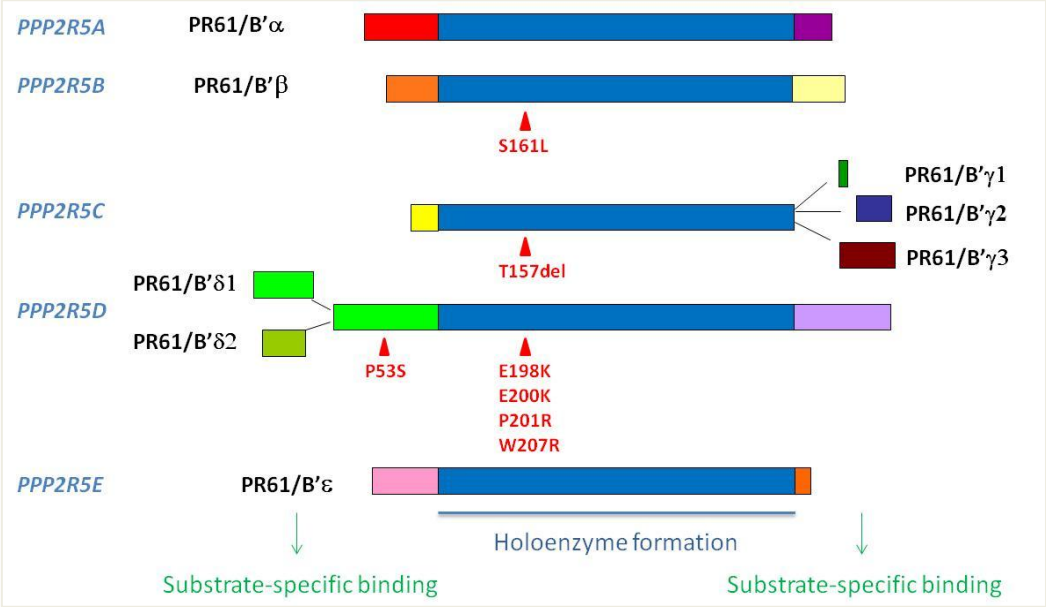

Figure 5: Representation of the B56/PR61/B' family showing the region responsible for holoenzyme formation and the predicted substrate binding domains. ID-associated mutations in the different subunit genes are indicated in red (arrowheads).

3. PP2A – directed therapy strategies

Because PP2A dysfunction and dysregulation have been implicated in numerous disease states, it has become a attractive, but difficult, therapeutic target. Depending on the setup, both PP2A inhibition as PP2A (re)activation seem to have a therapeutic potential. Several recent preclinical studies have shown that pharmacological reactivation of PP2A tumor suppressor activity by PP2A-activating drugs (PADs) indeed effectively antagonizes cancer development and progression [30, 116, 155]. Counter-intuitively, pharmacological inhibition of PP2A can equally-well cause cancer cell death, when combined with a DNA-damaging agent. This PP2A inhibitor-induced cell death is a result of mitotic catastrophe caused by premature entry into mitosis, deficient exit from mitosis or non-functional cell cycle checkpoints [30], highlighting the importance of PP2A holoenzymes in the regulation of cell division and DNA repair. The review papers in Chapters 3 and 4 briefly touch upon the PP2A-activating strategies used in the treatment of leukemia and the therapeutic opportunities of targeting the interactions between PP2A and cellular (oncogenic) inhibitors. Below, a more detailed and complete overview is provided on both PP2A-activating and -inhibiting therapeutic approaches.

3.1. PP2A reactivation

The first PAD to be tested was **forskolin**, a diterpene derived from *Coleus forskohlii* roots. This drug has the ability to induce adenylate cyclase and to activate PP2A independently of

adenylate cyclase stimulation. Later, the derivative **1,9-dideoxy forskolin** was designed, only harboring the PP2A activator function. *In vitro*, forskolin is able to suppress BCR-ABL – driven oncogenesis, causing apoptosis induction, reduced proliferation and impaired colony formation in chronic myeloid leukemia (CML), BCR/ABL-positive acute lymphoblastic leukemia (BCR/ABL^+-ALL) and acute myeloid leukemia (AML) [30, 155]. Moreover, treatment causes decreased leukemogenesis in immune-deficient mice injected with BCR/ABL^+ cells. Importantly, (1,9 dideoxy)forskolin does not affect $CD34^+$ normal cells, thus preventing toxicity on normal hematopoiesis [155].

Likewise, inhibitors of type 4 cyclic nucleotide phosphodiesterase (PDE4), like **rolipram**, elevate intracellular cyclic adenosine monophosphate (cAMP) levels in B-cell chronic lymphocytic leukemia (B-CLL), inducing apoptosis via the mitochondrial pathway by a mechanism involving PP2A activation and dephosphorylation of Bad [30].

Ceramide, a sphingolipid metabolite, inhibits proliferation and stimulates apoptosis in part due to activation of Ser/Thr phosphatases, in particular PP2A. It exerts anti-oncogenic effects in several prostate cancer cell lines, including androgen-independent ones characterized by decreased PP2A Cα expression. Interestingly, multiple promising chemotherapeutic agents are suspected or known to stimulate intracellular ceramide formation and subsequently induce apoptosis in a PP2A-dependent manner [30]. Ceramide has a direct effect on different PP2A holoenzymes *in vitro*, however it mainly affects the mitochondria-associated holoenzyme containing B'α in cell lines *ex vivo.* Recently, direct binding of ceramide to SET was demonstrated resulting in dissociation of SET-PP2A and PP2A activation [30].

A compound structurally related to ceramide is **FTY720** (Fingolimod, Gylenia™), one of the best studied PADs so far. FTY720 has been approved as immunosuppressant for the treatment of multiple sclerosis and for prophylaxis of solid organ transplantation rejection [156, 157]. This function is dependent on its phosphorylation. The PP2A activator function, however, is not. Therefore, non-phosphorylatable derivates were designed, like [S]-FTY720-OMe and OSU-2S FTY720. FTY720 shows a high oral bioavailability and lack of side effects [155]. Several studies evaluated FTY720 treatment for multiple leukemias (like B-CLL and multiple myeloma) and for bladder, breast, prostate and HCC, because of its ability to induce mitochondria-dependent apoptosis by interfering with Bcl2 and to suppress growth and survival signals by inhibiting ERK and PI3K/Akt pathways [116]. At least in leukemia, these effects can be attributed to PP2A activation. Mechanistically, doubt exists on the exact manner by which FTY720 activates PP2A. When administered to cells, FTY720 causes a transient activation of PP2A and results in dephosphorylation of Akt, Bad and p70S6 kinase, which all can be blocked by OA. Furthermore, FTY720 has been shown to activate a purified PP2A holoenzyme containing B55/PR55/B [158]. In leukemia, FTY720 (like ceramide) was shown to disrupt the SET–PP2A interaction causing PP2A reactivation [159]. FTY720's ability to antagonize leukemogenesis was demonstrated in BCR/ABL^+-ALL and B-CML, in which PP2A activity is inhibited through BCR/ABL-induced SET expression. Treatment resulted in inactivation/degradation BCR/ABL and inhibited survival signals mediated by Akt, ERK and Jak2 [116]. This anti-leukemic effect was observed in both imatinib (a BCR/ABL kinase

inhibitor)-sensitive and -resistant cells, without adverse effects on normal hematopoiesis. PP2A is also inactivated by the Jak2(V617F) oncogene in myeloproliferative neoplasms and this is mediated by SET. Consequently, FTY720 can down-regulate/inactivate Jak2 oncogene by binding to SET and in Jak2(V617F) leukemic mice this results in increased survival, decreased leukemic allelic burden and reduced splenomegaly, without apparent side effects [160]. FTY720 was also shown to have antileukemic effects in cell lines, *ex vivo* patient cells and animal models of BCR/ABL-negative lymphoid malignancies, like CLL, T-cell large granular lymphocyte leukemia, natural killer cell leukemia, mantle-cell lymphoma, and BCR/ABL^--ALL. However, whether the FTY720 anti-cancer effect is mediated by PP2A activation in these cases remains unclear [116]. Furthermore, for mutant c-Kit^+- AML *in vitro* or *in vivo* administration of FTY720 caused dephosphorylation of c-Kit and of its downstream effectors (Akt, ERK1/2 and STAT5), resulting in reduced proliferation, decreased clonogenic potential and apoptosis induction. The oncogenic c-KIT mutants in AML affect PP2A activity by reducing expression of various subunits, including A, PR55/Bα and PR61/B'αγδ [110]. Combined, these data show that PP2A inactivation (by BCR/ABL or Jak2(V617F) oncogene – induced SET expression or by c-Kit mutants) might be a key mechanism by which resistance to kinase inhibitor-based treatment is caused and leukemias are maintained, or even a key step in the cancer induction. Therefore, the possibility rises that patients could be brought into remission by kinase inhibitors and cured by FTY720 or derivatives, or that treatment with a combination of kinase inhibitors and FTY720 upon diagnosis might overcome therapeutic resistance or entirely avoid it.

To further target the SET-PP2A interaction, **SET binding peptides** COG112 and OP499 (formerly COG449) were designed, which are apolipoprotein E-mimetic peptides. In addition to disrupting the association of SET with PP2A, COG112 disrupts SET's interaction with the metastasis suppressor NM23-H1 and Rac1 in a glioma and breast cancer cell line, inhibiting cancer cell migration and invasion [161]. In CLL and non-Hodgkin lymphoma cells, OP449 downregulates the expression of the anti-apoptotic Bcl2 family member Mcl1 causing cytotoxicity *in vitro* and *in vivo* in tumor xenografts. Recently, OP449 treatment was shown to result in apoptosis of CML progenitors through a mechanism depending on PP2A reactivation. Combined with kinase inhibitors or chemotherapy, OP449 efficiently and selectively inhibited cell growth of leukemic cells, but not of normal cells.

Also CIP2A might be an attractive therapeutic target. Although mechanistically poorly understood, transcriptional downregulation of CIP2A by **rhabdocoetsin B**, a compound extracted from a traditional Chinese medicinal herb, induces apoptosis and Akt inactivation in a variety of lung cancer cells [30, 114]. Very similar, the proteasome inhibitor **bortezomib** down-regulates CIP2A in a dose- and time-dependent manner in bortezomib-sensitive liver cancer cell lines, resulting in Akt inactivation and apoptosis [30, 114]. The combination of bortezomib and sorafenib, a multi-kinase inhibitor, results in synergistic apoptotic effects in a CIP2A and PP2A-dependent manner. Furthermore, downregulation of CIP2A and subsequent PP2A mediated inhibition of Akt signaling can cause cancer cells to overcome resistance to CS-1008 (an anti-human TRAIL Death Receptor 5 antibody) or TRAIL-induced apoptosis [30, 114].

Recently, **phenothiazine** (PPZ), an FDA-approved neuroleptic type anti-psychotic drug, was demonstrated to activate PP2A in T-ALL cell lines accompanied by rapid dephosphorylation of multiple PP2A substrates (Akt, ERK, p70S6 kinase, c-Myc, and Bad), suppressed growth and apoptosis [162]. Since shRNA knockdown of the PP2Ac and A subunits attenuated the phenothiazine activity, PP2A mediates this antileukemic activity. In addition, PPZ treatment of a murine xenograft model of T-ALL diminished cell growth and led to dephosphorylation of Akt, p70S6 kinase, and Bad *in vivo*. Mechanistically, phenothiazine binds to the Aα scaffold subunit [162], but it remains unclear how it exactly stimulates PP2A activity. It has been suggested that PPZ interaction with Aα might have allosteric effects on PP2Ac activity or that PPZ might displace an A subunit regulator [163].

Several naturally occurring anti-oxidants have also been demonstrated to stimulate PP2A activity and induce apoptosis. **Dithiolethione** compounds inhibit proliferation in aggressive breast and lung cancer cell lines through PP2A-mediated downregulation of Akt, mTOR and c-Myc [30]. **Dihydroxyphenylethanol** promotes apoptosis in colon carcinoma cells by activation of PP2A, either direct or indirect via scavenging of reactive oxygen species known to inhibit PP2A [30]. Interestingly, an added pro-apoptotic effect in BCR/ABL$^+$ K562 cells was seen when combining the anti-oxidant **amifostine** and imatinib, compared to imatinib alone. This effect is in part due to transcriptional upregulation of *PPP2R5E* (encoding the pro-apoptotic B'ε subunit), suggesting that elevated expression of PP2A subunits may also be involved in anti-oxidant-mediated PP2A activation [30].

Finally, some other PADs are being evaluated as anti-cancer agents [116]: promethylating agents such as *Chloroethyl Nitrosourea* cause PP2A methylation and activation, leading to growth inhibition of melanoma cells; apoptosis of cancer cells is seen after α-Tocopheryl succinate treatment through a PP2A-dependent mechanism; PP2A activation by vorinostat and sorafenib induce gastrointestinal tumor-cell death; carnosic acid induces apoptosis of human prostate-carcinoma cells via PP2A-dependent effects on Akt/IKK/NFκB signaling; by upregulating the expression of PP2A regulatory subunits, methylprednisolone induces complete differentiation of leukaemic cells.

3.2. PP2A inhibition

One of the best studied pharmacological PP2A inhibitors is **fostriecin**, a natural compound with high selectivity for PP2A and PP4. Fostriecin directly binds to PP2Ac. It causes premature entry into mitosis resulting in potent and selective tumor cytotoxicity. However, due to relatively high instability, its clinical use is hampered [30].

Cytostatin, whose PP2A inhibiting mechanism is very similar to that of fostriecin, has anti-metastatic effects by blocking the adhesion of tumor cells to the extracellular matrix and by stimulating the expansion and activation of natural killer cells. However, also like fostriecin, cytostatin is instable [30].

Another PP2A inhibitor, **rubratoxin A**, possesses improved stability and shows anti-metastatic effects in mice. Unfortunately, hepatotoxicity is an issue [30].

Cantharidin is a toxin and anti-cancer agent. It is a non-specific PP2A inhibitor, since it also affects PP1. Cantharidin treatment results in apoptosis in different cancers, like in pancreatic cancer cell models via induction of caspase-8 and -9 and tumor necrosis factor α. In malignant testicular germ cell tumors, a combination of OA and cantharidin leads to apoptosis via MEK and ERK-mediated activation of caspase-3. Also in leukemia, PP2A inhibition results in apoptosis by activating caspases [164]. However, cantharidin has very toxic side effects preventing its clinical use. A less toxic demethylated analogue, **norcantharidin**, has a higher selectivity for PP2A while retaining the anti-cancer properties of cantharidin, as demonstrated in melanoma, breast, oral and gallbladder cancers and HCC [164].

Lactoferrin affects apoptosis-inhibiting PP2A complexes, like PP2Ac–α4. α4 is significantly upregulated in carcinogen-transformed human cells, HCC, breast cancer and small lung adenocarcinoma. Association of lactoferrin with α4 has pro-apoptotic and anti-oncogenic effects through reduction of α4-associated PP2A activity [30].

Lenalidomide is an FDA-approved drug for the treatment of a specific subset of myelodysplastic syndrome patients with an isolated deletion of chromosome 5q. It inhibits the PP2A Cα subunit resulting in cell cycle arrest and apoptosis, and has been shown to confer a significant increase in median overall survival in clinical trials. However, resistance can occur through overexpression of the PP2A Cα subunit [164].

Finally, the important role of PP2A in DNA damage and repair signaling might be exploited therapeutically by combining **PP2A inhibitors and DNA-damaging chemotherapeutic agents**. Indeed, LB1.2 (LB-102), a norcantharidin-like small molecule PP2A inhibitor, blocks the DNA-damage defense mechanisms induced by temozolomide (a DNA-methylating drug) or doxorubicin (a DNA-intercalating agent), leading to apoptotic cell death in cellular and mouse models of glioblastoma multiforme and neuroblastoma [30, 164]. It was predicted that such combination strategy might also be particularly beneficial to eradicate cancer cells of slow-growing and well-differentiated tumors or even quiescent 'cancer stem cells', both insensitive to cytostatic drugs [30]. Furthermore, **LB-100** is currently in phase 1 clinical trials examining the safety and efficacy of the small molecule PP2A inhibitor in the treatment of advanced solid cancers.

Despite the therapeutic promise of the above PP2A-targeting approaches, several challenges remain. It should be stressed that not all PP2A holoenzymes are tumor suppressive; some activate a certain signaling pathway, while others inhibit it. Thus, inhibiting or activating all forms of PP2A might not only be unnecessary for achieving the desired therapeutic effect, it may also pose serious risks for unwanted toxic side effects. Thus, targeting a specific holoenzyme or a specific PP2A-substrate interaction may be amongst the better future strategies to fully exploit the therapeutic potential of PP2A in human cancer or other diseases. In order to do so, more knowledge needs to be gained on the particular holoenzyme involved in a given pathway and the specific substrates of that holoenzyme. In addition, targeting a specific PP2A-inhibitor interaction, although challenging, remains a very attractive and cancer cell selective therapeutic approach [30].

2 Objectives

OBJECTIVES

PP2A represents one of the major protein phosphatase families in the cell, whose function is known to be disturbed by a variety of mechanisms in different pathologies, including Alzheimer's disease, diabetes, heart failure and cancer. Very recently, genetic screening and exome sequencing also implied PP2A dysfunction in ID. The **general aim** of my thesis is to biochemically and functionally characterize specific PP2A mutations, identified in patients with ID and endometrial cancer, of which the mechanism-of-action was still unknown. This seemed a particularly intruiging research question, as some of the involved mutations are surprisingly identical in both pathologies. With the obtained results, we eventually aimed to be able to suggest novel, potentially promising, therapeutic strategies.

Specific aims:

1. To firmly establish PP2A dysfunction as a novel cause for ID

Previously, the DDD study put forward *PPP2R5D* and *PPP2R1A* as novel candidate disease-causing genes [151]. Moreover, during the course of my thesis work, we became aware of more patients with different *PPP2R5D* and *PPP2R1A* mutations. In order to provide firm biochemical proof for causality and to identify the underlying mechanism by which PP2A dysfunction might affect signaling in the brain, we aimed to characterize the effects of B'δ and Aα mutations on PP2A holoenzyme formation and function. Strikingly, the ID-associated Aα mutations are identical to some found in serous endometrial cancer, highlighting the possibility of a common disease mechanism and rising the concern that ID patients harboring these mutations are at risk for developing cancer.

2. To biochemically and functionally characterize Aα mutations identified with high frequency in serous endometrial cancer

Mutations in *PPP2R1A* seem an important strategy of cancer cells to inactivate PP2A, and this occurs with high frequency in the more aggressive subtypes of endometrial cancer, i.e. serous endometrial cancer and carcinosarcoma. The reported Aα mutations are heterozygous, missense and show a high degree of recurrence. They cluster into HEAT-repeat 5 (P179L, P179R, R182W, R183G, R183Q, R183W) and HEAT-repeat 7 (R249H, S256F, S256Y, W257C, W257G, R258H), both believed to be involved in B-type subunit binding. However, the impact of these mutations on PP2A assembly and function was not investigated so far. We aimed to get a complete overview of their effects (1) on holoenzyme formation via cellular binding assays and (2) on the larger Aα interactome via an MS-based proteomics approach. In addition, we aimed to introduce these mutations in endometrial cancer cells and study their effect on cancer cell growth and oncogenic signaling. Eventually, our work should allow to provide a molecular basis for rational therapeutic intervention in this hard-to-treat cancer type, and thus, establish the *PPP2R1A* state (mutant or WT) as therapeutic biomarker.

3

Literature study (1):

Cellular inhibitors of Protein Phosphatase PP2A in cancer

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Cellular inhibitors of Protein Phosphatase PP2A in cancer.

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Abstract

Protein Phosphatases of type 2A (PP2A) represent a major class of structurally complex Ser/Thr phosphatases, which have recently gained a lot of interest in cancer biology because of their establishment as genuine tumor suppressors. PP2A phosphatases comprise a large family of >80 holoenzymes with complex structure, pleiotropic functions and diverse ways to regulate their biological activities. PP2A catalytic activity can be directly inhibited by an emerging set of specific cellular PP2A inhibitory proteins, including Acidic Nuclear Phosphoprotein 32a (ANP32a), Suvar 3-9/Enhancer of zeste/Trithorax (SET), Cancerous Inhibitor of PP2A (CIP2A), members of the cAMP-Regulated PhosphoProtein/α-Endosulfin (ARPP-16/19/ENSA) family, and Type 2A Interacting Protein (TIP). In addition, the PP2A Methyl Esterase 1 (PME-1), a regulator of reversible carboxy-terminal methylation of the PP2A catalytic C subunit, stabilizes an inactive PP2A C conformation and may be considered as an atypical inhibitor. Although sometimes poorly understood at the molecular level, these inhibitors either directly bind to the PP2A catalytic subunit or target very specific PP2A holoenzymes. In a wide variety of cancers, cellular PP2A inhibitors are aberrantly expressed, sometimes with very high frequency, and this constitutes a major mechanism by which the tumor suppressive function of PP2A can be impaired within a tumor. This offers interesting perspectives for therapeutic interference and some promising pre-clinical studies will be discussed.

Keywords: ANP32a, ARPP-16/-19, CIP2A, ENSA, phosphatase, PME-1, PP2A, SET, TIP, tumor suppressor

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Introduction

Constitutive activation of oncogenic kinases is one of the hallmarks of cancer cells, driving uncontrolled cell proliferation, invasion and potentially metastasis [1]. Therefore, it should come of little surprise that tumor suppressive functions have been attributed to many protein phosphatases, the natural kinase antagonists [2,3]. In particular, the Ser/Thr phosphatases of type 2A (PP2A) are currently recognized as major tumor suppressors, counteracting specific signaling pathways driving survival, proliferation and cell migration [reviewed in 4,5,6,7,8,9,10,11]. In addition, PP2A phosphatases are important physiological regulators of proper checkpoint functioning during the cell cycle, which contributes significantly to their tumor suppressive abilities [reviewed in 12,13,14,15]. However, not all PP2A complexes counteract cancer promoting pathways, but conversely, rather help sustaining them [reviewed in 10,16]. These seemingly contradictory observations can mainly be explained by the enormous diversity of PP2A holoenzymes within the cell (Fig 1). Indeed, in humans PP2A enzymes comprise an extended family of >80 holoenzymes with complex structure, pleiotropic functions and diverse ways to regulate their catalytic and biological activities [reviewed in 17]. The core structure consists of a catalytic subunit (PP2A-C) and a 65 kDa structural subunit (PR65 or A subunit), each encoded by two different genes (giving α and β isoforms). This dimeric PP2A AC core can exist as such, but is in the majority of cases associated with a third variable regulatory B-type subunit. These B-type subunits (at least 20 in humans) act as targeting and substrate specifying entities, thus determining the physiological functions and regulation of the trimeric complexes (Fig 1). In addition, there is evidence for the existence of some 'atypical' PP2A complexes devoid of any A or B-type subunits, but comprising the α4 protein (also called IGBP1 or Tap42 in yeast) instead [18,19,20] (Fig 1). The A subunit and α4 interact in a mutually exclusive way with PP2A-C [21,22]. Binding of α4 allosterically modifies the PP2A-C active site [23], thus giving rise to the particular substrate specificity of the α4–C complex [24]. In summary, the

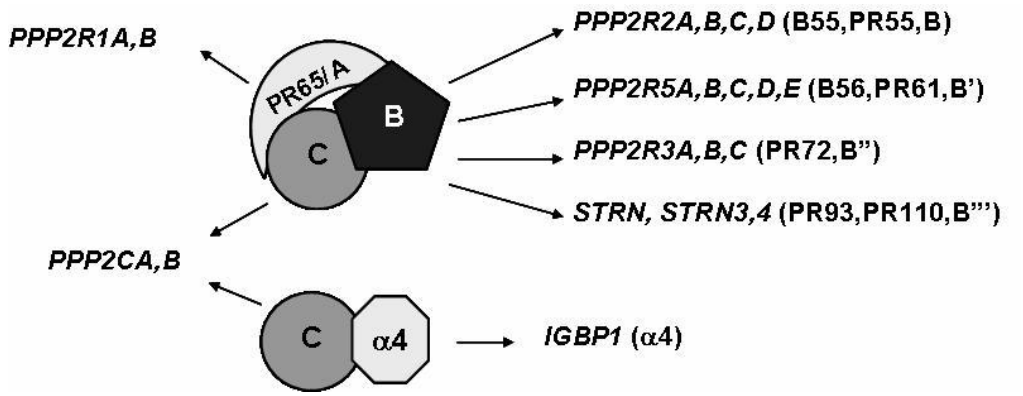

Figure 1. *PP2A holoenzyme structure*
The majority of PP2A complexes within a cell are heterotrimers, consisting of a catalytic C, a structural A and one regulatory B-type subunit. In humans, the C and A subunits are each encoded by two different genes (indicated in italics), giving rise to an α and β isoform. For the B-type subunits 15 human genes (indicated in italics) have been described, giving rise to >20 isoforms belonging to four different families, PR55/B (or B55), PR61/B' (or B56), PR72/B" and PR93/PR110/B"'. A smaller fraction of PP2A exists as the core AC dimer, or as an α4–C complex. This enormous structural diversity creates specificity and forms the basis for PP2A's pleiotropic physiological functions.

combinatorial assembly of all these multimeric PP2A holoenzymes provides the essential determinants for substrate specificity, subcellular targeting and fine-tuning of phosphatase activity [25,26,27], explaining the pleiotropic and sometimes opposing functions of PP2A within cells.

The tumor suppressive PP2A complexes mainly appear to be constituted of PR61/B' (or B56) subunit family members [5,8,9,10,11], but surprisingly hardly any mutations have been found in these or in fact any of the B-type or C subunit encoding genes in human tumor patient samples. Instead, inactivating mutations and deletions in both *PPP2R1A* and *PPP2R1B* occur with low frequency in several human cancers, identifying Aα and Aβ as genuine tumor suppressors [6]. For Aα these cancer-associated subunit mutants show impaired interaction with the C subunit and/or with particular B-type subunits, most notably those of the PR61/B' class [28,29], whereas Aβ mutants also show impaired interaction with particular PP2A substrates, most notably the small GTPase RalA [30]. This results in RalA hyperphosphorylation and activation [30], or in impaired dephosphorylation of various PR61/B'-containing PP2A substrates, including the survival kinase Akt, proto-oncogen c-Myc, tumor suppressor p53 and some apoptotic regulators of the Bcl-2 family [8-11].

Another emerging mechanism by which the tumor suppressive activity of PP2A can be disturbed in human tumors is through aberrant expression of specific cellular PP2A inhibitors [9,11,31]. As we will discuss in this review, this occurs in a wide variety of cancers, often with high frequency and correlating with cancer stage and therapeutic outcome. We will highlight the mechanism-of-action, function and regulation of these important PP2A regulators, and eventually we will discuss how this knowledge might be exploited for therapeutic purposes.

PP2A Inhibitor-1 and Inhibitor-2 (ANP32a and SET)
Historically, PP2A Inhibitor-1 (I_1^{PP2A}, also called ANP32a or PHAP-I or pp32 or LANP or mapmodulin) and Inhibitor-2 (I_2^{PP2A}, also called SET or PHAP-II or TAF-Iβ) were the first cellular PP2A inhibitors to be discovered [32,33,34]. Both proteins potently inhibit PP2A with IC50's in the low nM range in a non-competitive manner, likely through direct binding to the PP2A C subunit [33,34]. At the same time, in the presence of near-physiological concentrations of Mn^{2+}, both PP2A inhibitors can stimulate PP1 activity towards some substrates, suggestive for a potential role in coordinating cellular PP2A and PP1 activities [35].

ANP32a and ANP32e
ANP32a belongs to a large family of at least 9 members (denoted ANP32a-h) sharing a highly acidic C-terminal domain and a Leucine Rich Repeat (LRR)-containing N-terminal domain [36,37] (Fig 2A). So far, only the a and e isoforms show PP2A inhibitory ability in an *in vitro* phosphatase assay [34,38,39] and their third LRR might be required for this [38]. Since this domain is well-conserved in some other ANP32 isoforms (Fig 2A), it can not be excluded that yet other ANP32 members might show PP2A inhibitory activity as well. Notably, the LRR domains might be involved in homo- or heterodimerisat-

ion of ANP32a/e [39,40] leaving open the possibility this would be a prerequisite for their PP2A inhibitory effect. Both ANP32a and ANP32e are phosphoproteins [39,40,41]. Jacalin, a dietary lectin with anti-proliferative effect, induces tyrosine phosphorylation of ANP32a on a not yet identified site and this releases the association with PP2A, allowing increased PP2A activity and inactivation of the ERK signaling pathway [42]. In addition, ANP32a Ser-158 and Ser-204 were identified as constitutive *in vivo* casein kinase 2 phosphorylation sites [43], so far with unknown function. Phosphorylation of ANP32e was shown to be required for the ANP32e–PP2A interaction [39], but the molecular basis for this remains poorly understood. ANP32a and ANP32e are found both in the cytoplasm and the nucleus, reflecting their nucleocytoplasmic shuttling behavior that is regulated by the presence of a classical Nuclear Localization Signal (NLS) and two Nuclear Export Signals (NES) [36,38,39,40,41,44] (Fig 2A).

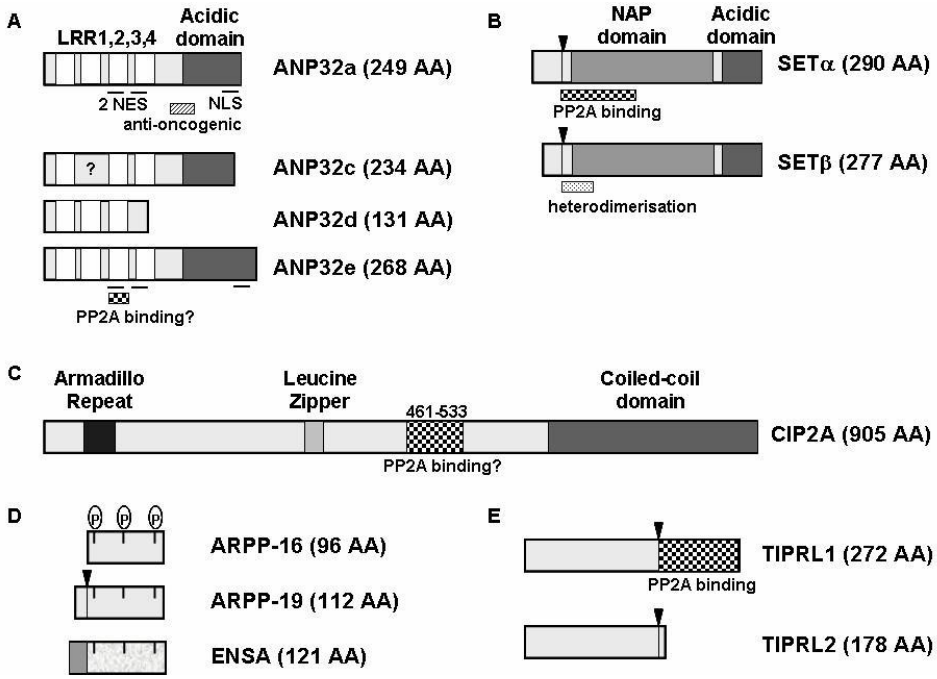

Figure 2. *Structural and functional domain organization of PP2A inhibitory proteins*
A. *ANP32 family members. The four indicated isoforms, a, c, d and e, are encoded by different genes. The Leucine-Rich Repeats (LRR), Nuclear Export Signals (NES) and Acidic domain are indicated. PP2A inhibition has only been shown for ANP32a and e through a putative PP2A binding domain located in LRR3. ANP32c and d miss the C-terminal Nuclear Localization Signal (NLS).* ***B.*** *SET isoforms. Both splice variants, SETα and SETβ, bind and inhibit PP2A, without a need for dimerisation. The Nucleosome Assembly Protein (NAP) and Acidic domains are indicated.* ***C.*** *CIP2A domain structure. A deletion mutant that lacks amino acids 461-533 shows reduced PP2A binding.* ***D.*** *ARPP-16/19/ENSA family members. ARPP-16 and -19 are splice variants of a single gene, while ENSA is encoded by a different gene. Three highly-conserved phosphorylation sites are indicated, resp. for CDK, Greatwall/MASTL kinase and PKA.* ***E.*** *TIP isoforms. Only the longest splice variant, TIPRL1, can bind and inhibit PP2A.*

ANP32a has multiple cellular functions, and it is currently unclear to what extent inhibition of PP2A contributes to all these diverse roles. As such, ANP32a was essentially identified as a tumor suppressor, inhibiting oncogene-induced cell transformation [45] through a region encompassing amino acids 150-174 [46]. As this region does not coincide with the putative PP2A binding domain, this could suggest the negative role of ANP32a in cell transformation might be independent of PP2A inhibition (Fig 2A). In addition, this region is absent in ANP32d and somewhat divergent with the corresponding sequences in ANP32c and ANP32e (Fig 2A), providing a putative explanation for the oncogenic roles of these three ANP32 isoforms [46,47,48]. In line with a tumor suppressive role, ANP32a downregulation results in phenotypic changes associated with cell transformation, while ectopic expression of ANP32a abolishes Ras-mediated transformation *in vitro* and tumorigenesis *in vivo* [49]. These effects may occur in part through negative regulation of the Raf-1/MEK/ERK pathway [50]. In accordance, in pancreas and prostate cancer ANP32a expression is severely reduced [51,52]. ANP32a is also strongly pro-apoptotic by accel-

erating caspase-9 and subsequent caspase-3 activation after apoptosome formation [53,54]. In some cancer cells, ANP32a-mediated apoptosis can specifically be inhibited through physical interaction with the hyperphosphorylated form of the retinoblastoma tumor suppressor protein [55]. Decreased ANP32a expression in non-small-cell lung cancer is associated with apoptotic resistance [56]. As part of the SET complex (an ER-associated complex of five proteins including SET), ANP32a also promotes caspase-independent granzyme A-mediated cell death. Intriguingly, SET inhibits this process by inhibiting the DNase nicking activity of the tumor metastasis suppressor NM23-H1, another one of the five proteins constituting the SET complex [57]. This suggests that in contrast to ANP32a, SET rather has oncogenic properties. ANP32a is also involved in the regulation of mRNA trafficking as part of the HuR complex, which stabilizes and transports short-lived mRNAs of particular proto-oncogenes and stress response proteins from the nucleus to the cytoplasm [58]. Under lethal stress conditions, ANP32a-mediated cytoplasmic translocation of HuR is promoted and this contributes to the apoptotic response [59]. ANP32a and SET both also function in transcription regulation and chromatin remodeling as parts of the INHAT (INHibitor of Acetyl Transferase) complex that binds to histones and regulates histone modifications [60]. Both SET and ANP32a directly bind to basic N-terminal histone tails, particularly when the latter are not phosphorylated or hyperacetylated, and this is thought to inhibit gene transcription by a histone masking mechanism that prevents acetyltransferase access [61,62,63]. In addition, SET and ANP32a actively recruit transcription inhibiting histone deacetylases [63]. ANP32a may also repress transcription of specific genes through binding the transcriptional repressor E4F and modulating its activity [64]. Ataxin 1, a protein mutated in SpinoCerebellar Ataxia type 1 and a known interaction partner of ANP32a [65], relieves this repression by competing with E4F for ANP32a [64]. By affecting expression of the neurofilament light chain gene ANP32a may play a role in the epigenetic modulation of neuronal differentiation [66]. Finally, a structural role for ANP32a in cytoskeletal dynamics and microtubule- and dynein-dependent organelle transport has also been inferred, particularly in neurons, through its interaction with Microtubule-Associated Proteins (MAPs) [36,40,67]. For effective interaction with MAPs, ANP32a phosphorylation might be required [40].

Despite all these diverse cellular functions, ANP32a-null mice are without any apparent phenotype [68], possibly reflecting functional redundancy provided by other members of the ANP32 family. Likewise, ANP32e-null mice show no apparent abnormalities except for a very subtle neurological clasping and mild motoric phenotype [69,70], in line with its presumed role in synaptogenesis [38,39]. In contrast, ANP32b knockout mice show perinatal lethality due to variable defects in various organ systems, demonstrating some hierarchy of importance in mammalian ANP32 genes [71].

SET

Although SET does not structurally belong to the ANP32 family, it also harbors a highly acidic C-terminal domain [44,72]. Two SET splice variants exist, each with a different N-terminal region of 39 (α isoform) or 26 (β isoform) amino acids encoded by alternative first exons [73] (Fig 2B). While the Nucleosomal Assembly Protein (NAP) domain is essential for SET's chromatin remodeling and histone chaperone activity [73,74], the PP2A inhibitory activity resides within a 95-amino-acid-region immediately C-terminally of the alternative N-terminal regions (Fig 2B), explaining why both SET isoforms can inhibit PP2A [75]. Overexpression of SET induces increased expression, phosphorylation, DNA-binding and transcriptional activity of c-jun, and this is reversed by ectopic expression of PP2A C, consistent with SET acting as a PP2A inhibitor in cells [76].

SETα and SETβ have originally been identified as the host factors that promote adenoviral DNA replication, nucleosome assembly and transcription through chromatin remodeling [73,77], an activity that requires their C-terminal acidic domain and their heterodimerisation through a coiled-coil domain [78] (Fig 2B). SET dimerisation is however unnecessary for inhibition of PP2A [75]. Like ANP32a, SET is a phosphoprotein, and Ser-9 and Ser-24 in SETβ have been identified as potential protein kinase C sites [79]. Using phospho and non-phospho mimicking mutants, phosphorylation of Ser-9 is predicted to prevent SETβ dimerisation, while it is mandatory for PP2A inhibition [80]. SET is found in the cytoplasm but also in the nucleus, potentially as a C-terminally truncated form or as part of a SET homo- or ANP32a–SET heterodimer within larger complexes such as the INHAT and SET complex [44,57,60,62,63,79,81,82]. It is quite intriguing to note that ANP32a and SET can function both opposite to each other [57] as well as in conjuction with each other [60] within different of these multiprotein complexes. Next to its role as a transcriptional repressor within the INHAT complex, some reports have identified SET as a transcriptional corepressor of specific transcription factors, including some members of the nuclear receptor superfamily [83,84,85] and even as a genuine DNA-binding transcription factor [86,87].

SET expression is modulated as a function of cell proliferation, with high expression in rapidly dividing cells or transformed cells, and low expression in quiescent or contact-inhibited cells [82,88,89]. SET can interact with the cyclin-dependent kinase (CDK) inhibitor p21/Cip1, cyclin B and glyceraldehyde-3-phosphate dehydrogenase and these interactions contribute to its regulatory role in the

cell cycle at the G2/M transition [90,91]. SETβ also plays a positive role in cell migration and is recruited to the cell membrane of migrating cells in a Rac1-dependent way, requiring Ser-9 phosphorylation of SET [80]. This might lead to local amplification of Rac1-stimulated kinase signaling by preventing PP2A activity towards these Rac1 downstream kinases. In accordance with these inferred pro-oncogenic and pro-migratory roles, SET overexpression has been observed in human patient samples of Wilms' tumour [88], hepatoma [92], choriocarcinoma [93] and several types of leukemia including Bcr/Abl (Philadelphia chromosome)-positive blast crisis chronic myelogenous leukemia (bc-CML) [94], Bcr/Abl-positive acute lymphocytic leukemia (ALL) [95], acute myeloid leukemia (AML) [96] and B-cell chronic lymphocytic leukemia (B-CLL) [97]. Restoration of PP2A activity by PP2A-C overexpression or shRNA-mediated knockdown of SET in these leukemia cells has a therapeutic effect [94,97] and results in decreased levels of hyperphosphorylated Rb and Myc, suppression of STAT5, ERK1/2, Akt, Bad and Jak2 phosphorylation, and induction of SHP-1-mediated Bcr/Abl dephosphorylation (inactivation) and degradation [94].

In acute undifferentiated leukemia (AUL), AML and T-cell ALL (T-ALL), the SET gene is involved in recurrent chromosomal rearrangements and translocations, in particular with the nucleoporin Nup214 gene (also called CAN) [98,99,100,101]. In all these cases this gives rise to an identical chimeric SET-Nup214 fusion protein with altered, exclusively nuclear subcellular localization that is causally involved in the overall impairment of NES-mediated nuclear protein export [102,103], implying that not an altered SET but rather an altered Nup214 function contributes to the leukemogenic process. In the T-ALL LOUCY cell line the SET-Nup21 fusion, but not SET itself, activates *HOXA* gene expression, increases cellular proliferation and inhibits differentiation [101]. The latter was confirmed in transgenic mice expressing SET-Nup214 in the hematopoietic lineage as these mice show impaired differentiation of erythroid, megakaryocytic and B-cell lineages [104]. Likewise, in another transgenic SET-Nup214 model an expansion of early hematopoietic progenitors and a partial depletion of lymphocytes were observed [105]. None of these transgenic models were however leukemia-prone, suggesting the fusion protein is incapable of inducing transformation on its own and additional genetic aberrations might be necessary for disease development [104,105].

Cancerous Inhibitor of PP2A (CIP2A)

CIP2A is a cellular PP2A inhibitor with oncogenic activity that was originally identified as a novel coprecipitating partner of the PP2A A subunit [106]. Structurally, it contains an Armadillo Repeat, Leucine Zipper and Coiled-Coil domain of unknown function, while the PP2A binding domain comprises at least amino acids 461-533 (Fig 2C). Functionally, CIP2A directly interacts with the c-Myc oncogene, inhibits cellular PP2A activity toward c-Myc Ser-62 and thereby prevents c-Myc proteolytic degradation [106]. This implies CIP2A might specifically target the PR61/B'α-containing PP2A holoenzyme that is known to dephosphorylate c-Myc at this site [8], but exactly how this occurs remains largely unknown. In addition to regulation of c-Myc phospho-status, CIP2A was identified as a major player in dependence receptor signaling by inhibiting UNC5H2-dependent apoptosis [107] and in phosphatidylinositol 3-kinase-mediated survival signaling by inhibiting Akt kinase-associated PP2A activity [108]. These two functions clearly also contribute to its oncogenic characteristics. Notably, CIP2A is barely detectable in normal cells, and becomes specifically upregulated in a very significant percentage (40-87%) of tumor patient samples of various origins, including head and neck cancer [106], colon cancer [106], gastric cancer [109,110,111], tongue cancer [112], liver cancer [110], lung cancer [113,114,115], esophageal cancer [110,116], AML [96,117], CML [118], prostate cancer [119], breast cancer [120], serous ovarian cancer [121] and cervical cancer [122]. In addition, its expression correlates with poor prognosis and cancer aggressiveness [109,112,113, 118,120,121]. While functional SNPs at the CIP2A promoter or promoter methylation are unlikely to account for regulating CIP2A expression in cancer cells [123], CIP2A transcription was found to be regulated by c-Myc in a positive feedback loop, as depletion of either of these two oncoproteins inhibits the expression of the other [109]. In cancer cells with increased EGFR-MEK1/2-ERK pathway activity, ETS1 is involved in mediating increased CIP2A transcription [123].

PP2A inhibitors of the ARPP-16/-19/ENSA family

cAMP-Regulated PhosphoProteins ARPP-16 and ARPP-19 are splice variants and members of an evolutionary conserved protein family with multiple isoforms, initially discovered in brain as substrates of PKA *in vitro* and *in vivo* [124]. ENSA (α-endosulfine) is closely related to ARPP-19, but is generated from a distinct gene and mainly differs within its 20-amino-acid N-terminal domain (Fig 2D). ENSA was originally named after its identification as a putative endogenous ligand of the sulfonylurea receptor K^+ channels in pancreatic β cells [125]. ARPP-19 and ENSA have recently come into the picture as the long sought-after mitotic PP2A inhibitors in *Xenopus* oocytes that promote the G2/M transition and the mitotic state [126,127].

Both proteins require phosphorylation of a conserved serine residue (Ser-67 in ENSA and Ser-62 in ARPP-19) by the Greatwall kinase in order to bind PP2A and subsequently inhibit PP2A activity towards a physiological CDK1 substrate. In accordance, ENSA (also called En-

dos) mutant oocytes in *Drosophila* have a prolonged prophase and fail to progress to metaphase [128], while a Greatwall loss-of-function allele likewise leads to cell cycle delay at the G2/M transition [129]. A similar role for ARPP-19/ENSA and the mammalian Greatwall orthologue MAST-like kinase have been implicated in the regulation of mitosis in HeLa cells [127,130], suggesting evolutionary conservation of this regulatory circuit. In line with a critical function for the protein(s), ARPP-16/19 knockout mice exhibit embryonic lethality [131]. The particular PP2A holoenzyme that needs to be inhibited to allow mitotic entry, and subsequently needs to be reactivated to dephosphorylate CDK1-cyclin B substrates and allow mitotic exit, harbors a regulatory PR55/B subunit (PR55/Bδ in *Xenopus*, Twins/B55 in *Drosophila* and PR55/Bα or δ in mammalian cell lines) [132,133,134, 135,136]. Notably, Greatwall phosphorylated ARPP-19/ENSA strongly bind PP2A-PR55/Bδ from interphase extracts, but no other B-type subunits or other Ser/Thr phosphatases. In addition, they do not inhibit the monomeric C subunit or the dimeric AC form of PP2A, suggesting they might be highly specific inhibitors of PP2A-PR55/Bδ [126,127]. If and how their well-conserved CDK and PKA sites (Fig 2D), that can be robustly phosphorylated by these kinases *in vitro* [126], might contribute to PP2A regulation remains to be determined.

Type 2A Interacting Protein (TIP)

The Tip41 protein was originally discovered in yeast as a Tap42/α4 interaction partner [137]. In yeast two-hybrid assays, like Tap42, Tip41 (bait) was shown to interact with all PP2A-like yeast phosphatases including the orthologues of PP2A, PP4 and PP6 [138]. In mammalian cells however, TIP (also called hTip41 or TIPRL isoform 1) does not directly bind α4 but rather primarily interacts with the PP2A, PP4 or PP6 C subunits [138,139,140]. *In vitro* and *in vivo* binding assays have supported this view, demonstrating that TIP does not compete with α4 for PP2A-C binding, and that even a trimolecular TIP–C– α4 complex can be formed [139]. TIP is a ubiquitously expressed PP2A inhibitory protein [139,140], and like α4, its effect on PP2A activity was proposed to be allosteric in nature as okadaic acid, a PP2A C catalytic site binding inhibitor, failed to dissociate the TIP–PP2A-C inte raction [139,140]. The PP2A inhibitory activity resides in the C-terminal part that is lacking in the shorter TIP splice variant TIPRL2 (also called TIP_i2) which does not inhibit PP2A *in vitro* [140] (Fig 2E). Notably, TIP may play an important role in DNA damage and repair signaling as it regulates PP2A enzymes that oppose ATM/ATR-dependent phosphorylation events [140] and is part of a well-defined yeast PP4 holoenzyme, which is involved in regulating cisplatin sensitivity [138]. These observations definitely warrant further addressing the TIP status in cancer cells, which is currently still unknown.

The PP2A methylesterase PME-1: an atypical PP2A 'inhibitor'

The highly conserved C-terminal tail of the PP2A C subunit is post-translationally modified by a methyl esterification of the free carboxy-terminus of the C-terminal Leu -309 residue. This reaction is catalyzed by an S-adenosylmethionine (SAM)-dependent methyltransferase LCMT1 [141] and the PP2A methylesterase PME-1 [142], both essential enzymes for maintaining mammalian cell and organism viability [143,144,145]. PP2A methylation greatly facilitates PP2A holoenzyme assembly with specific regulatory B-type subunits, particularly those of the PR55/B family, the binding of which absolutely requires this modification [reviewed in 146]. These observations are underscored by several structural studies [reviewed in 26]. LCMT1 activity and expression can be regulated by changes in homocysteine metabolism, which itself is affected by dietary intake of folate and vitamins B6 and B12 [147]. Elevated plasma levels of homocysteine can lead to decreased PP2A methylation and impaired holoenzyme assembly with PR55/B subunits, contributing to conditions such as Alzheimer's Disease [147] and potentially cancer [148]. However, whether PP2A-C methylation might be affected in human tumors remains to be established.

In addition to its methylesterase activity, PME-1 has been attributed yet another PP2A regulatory function as it can bind and stabilize a native inactive PP2A form [149], estimated to represent up to 20-25% of the total cellular PP2A pool [150]. This inactive PME-1-bound PP2A can be (re)activated by an ATP/Mg^{2+}-dependent PP2A activating enzyme PTPA (Phosphatase Two A Phosphatase Activator) [149], originally discovered as an activator of PP2A tyrosyl phosphatase activity *in vitro* [151] but subsequently characterized as a prolyl peptidyl *cis/trans* isomerase with Pro-190 of the PP2A C subunit as its unique target [152,153]. This regulatory PTPA–PP2A–PME-1 circuit constitutes part of the PP2A biogenesis process, as PP2A-C was found to be translated as an inactive form that subsequently needs to be assembled into active PP2A trimers in a highly regulated manner [154,155]. In order to prevent any promiscuous activity of free C subunit in the course of this process, PME-1 apparently stabilizes an inactive PP2A dimer until it is activated by PTPA, methylated by LCMT1 [156] and eventually assembled into a functional holoenzyme with restricted catalytic activity due to the presence of the regulatory B-type subunit. Thus, unlike genuine PP2A inhibitors, PME-1 is unable to inactivate active PP2A holoenzymes [143], but rather seems to act as a stabilizer of an inactive PP2A form, in a manner that may be even unrelated to its methylesterase activity [157]. Notably, in some cancer cells this PME-bound, inactive PP2A fraction might be significantly increased as in glioblastoma

PME-1 overexpression correlated with tumor staging and increased activity of the MEK/ERK pathway [158].

Concluding remarks: therapeutic targeting of cellular PP2A inhibitors in cancer

In summary, many of the PP2A inhibitors described above are clearly aberrantly expressed in various cancers and this may contribute significantly to tumorigenesis and tumor progression (Fig 3). While most inhibitors, such as SET or CIP2A act as oncoproteins, in line with the well-established tumor suppressor functions of PP2A, some inhibitors, such as ANP32a, are tumor suppressive themselves, potentially reflecting they might act on different PP2A holoenzymes. At least for the oncogenic PP2A inhibitors, it seems a valuable therapeutic strategy to attempt interfering with their increased expression in cancer cells, or else relieving somehow their PP2A inhibitory mode-of-action. Such approaches can indeed be expected to restore PP2A's anti-proliferative and pro-apoptotic functions, and ultimately block tumor growth and induce apoptotic death. In fact, several recent pre-clinical studies have demonstrated the feasibility of such strategies [reviewed in 11,31].

Although mechanistically poorly understood, transcriptional downregulation of CIP2A by rhabdocoetsin B, a compound extracted from a traditional Chinese medicinal herb, induces apoptosis and Akt inactivation in a variety of lung cancer cells [159]. Likewise, bortezomib, a proteasome inhibitor, down-regulates CIP2A in a dose- and time-dependent manner in bortezomib-sensitive liver cancer cell lines, resulting in Akt inactivation and apoptosis [160]. The combination of bortezomib and sorafenib, a multi-kinase inhibitor, results in synergistic apoptotic effects in a CIP2A/PP2A-dependent manner [161]. In addition, CIP2A downregulation and subsequent PP2A-mediated inhibition of Akt signalling can sensitize cancer cells to TRAIL-induced cell death [162,163]. Along the same lines, reduction of SET levels has been demonstrated following imatinib (a Bcr/Abl kinase inhibitor) and Janus kinase 2 inhibitor (HBC or AG490) treatment in CML cell lines and mouse cells expressing imatinib-resistant forms of Bcr/Abl, presumably also through a transcription-dependent mechanism [94,164].

Direct therapeutic interference with the PP2A–SET interaction was highlighted in two reports in which SET was identified as a novel cellular binding target of COG112 [165,166], an apolipoprotein E-mimetic peptide, that targets the oncogenic functions of SET in a glioma and breast cancer cell line by disrupting its association with three different cellular SET targets: PP2A, the metastasis suppressor NM23-H1 and Rac1 [166]. The resulting increase in PP2A activity corresponded to decreased Akt signalling and c-Myc stability [166], while in immune cells decreased NF-κB signalling, phospho-p38 and phospho-Akt were observed [165]. In both systems this resulted in potent anti-proliferative and pro-apoptotic effects. Likewise, treatment of primary B-CLL cells with COG449, a dimerised derivative of COG112, increased PP2A activity and induced cytotoxicity *in vitro* and *in vivo*, while normal B cells were 2-log units less sensitive, indicative for cancer cell specificity [97].

Another compound that might directly interfere with SET-mediated inhibition of PP2A, is FTY720 (Fingolimod), a sphingosine-like molecule, recently approved for multiple sclerosis treatment. FTY720 induces mitochondria-dependent apoptosis and has potent anti-proliferative effects, particularly in several leukemias [31,94,95,167, 168,169]. The underlying mechanism involves a direct increase of PP2A activity, which was originally low due to increased bcr/abl-induced expression of SET. FTY720-mediated PP2A activation might involve intracellular production of ceramide and subsequent ceramide-induced dissociation of the PP2A–SET interaction [170]. In mutant c-KIT-positive AML however, FTY720 also increased inhibited PP2A activity, which was in this case low by c-kit-induced reduced expression of various tumor suppressive PP2A subunits, including Aα and PR61/B'α or γ [171], indicative for other cellular FTY720 targets besides SET. In any case, from these studies it was suggested that reactivation of PP2A might prove particularly therapeutically beneficial in those cases where oncogenic tyrosine kinase mutations, such as bcr/abl and mutant c-kit, underlie resistance to current kinase inhibitor therapies [171].

In conclusion, the aforementioned studies clearly have demonstrated the therapeutic promise of interfering with oncogenic PP2A inhibitors, such as CIP2A and SET, in several human cancers. However, in order to further develop these SET or CIP2A antagonizing molecules into drug-like compounds, it will be of utmost importance to improve our current insights into the molecular and biochemical mechanisms by which these inhibitors target PP2A or specific PP2A holoenzymes and inhibit PP2A activity. For this purpose, structural studies are urgently needed. All this essentially also applies for the more recently discovered members of the ARPP-16/-19/ENSA family and TIP. In these cases, additional studies are required to investigate their status in human tumor samples and cancer cells. For PME-1 highly specific esterase catalytic inhibitors have been reported [172,173], but it remains to be determined how beneficial they might be to stop proliferation or induce apoptosis, for instance in cellular models of glioblastoma. It is in any case expected that studying these cellular PP2A inhibitors will only gain importance in current cancer biology and drug discovery research.

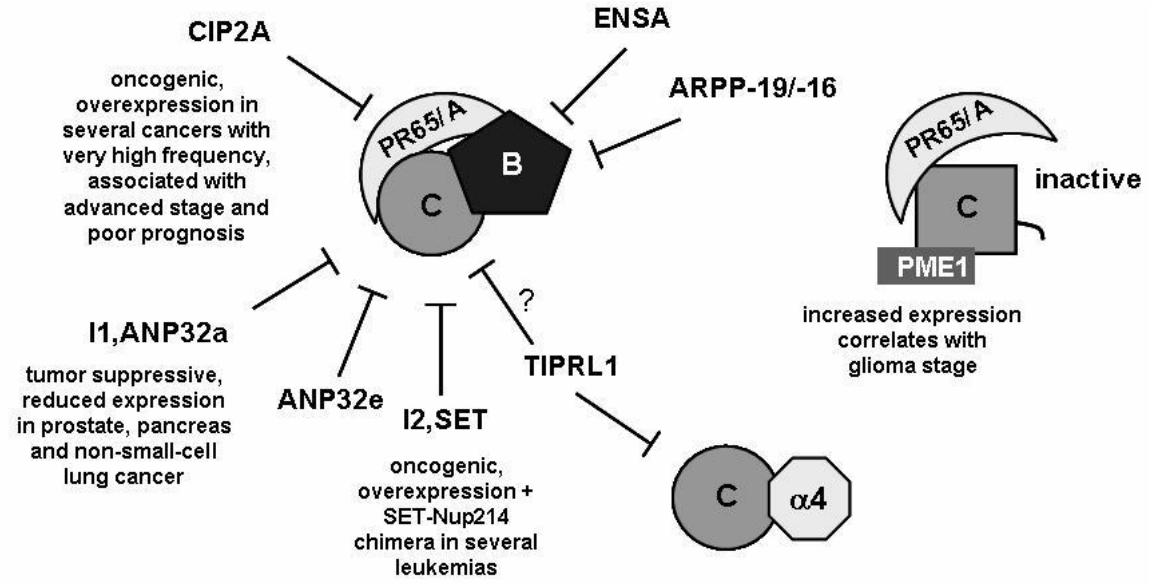

Figure 3. *Cellular PP2A inhibitors and their status in cancer cells*
Currently well-established PP2A inhibitory proteins are depicted alongside the PP2A subunit which they presumably target: while TIPRL1, SET and ANP32a/e have clearly been shown to directly bind to PP2A-C, the PP2A binding partner of CIP2A is not yet entirely clear, although CIP2A was found in immunoprecipitates of the A subunit. The mitotic inhibitors ENSA and ARPP-19 target a specific B-type subunit, PR55/Bδ, and this requires their prior phosphorylation by Greatwall/MASTL kinase. The inactive PP2A–PME-1 complex is depicted separately to indicate that although PME-1 has a high affinity for an inactive PP2A-C conformation, it can likely not actively inhibit PP2A. For some of the inhibitors their status in cancer cells is summarized; as far as we know, any abnormalities have not yet been described for TIPRL or the ARPP-19/ENSA members.

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4

Literature study (2):

The basic biology of PP2A in hematologic cells and malignancies

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The basic biology of PP2A in hematologic cells and malignancies

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Reversible protein phosphorylation plays a crucial role in regulating cell signaling. In normal cells, phosphoregulation is tightly controlled by a network of protein kinases counterbalanced by several protein phosphatases. Deregulation of this delicate balance is widely recognized as a central mechanism by which cells escape external and internal self-limiting signals, eventually resulting in malignant transformation. A large fraction of hematologic malignancies is characterized by constitutive or unrestrained activation of oncogenic kinases. This is in part achieved by activating mutations, chromosomal rearrangements, or constitutive activation of upstream kinase regulators, in part by inactivation of their anti-oncogenic phosphatase counterparts. Protein phosphatase 2A (PP2A) represents a large family of cellular serine/threonine phosphatases with suspected tumor suppressive functions. In this review, we highlight our current knowledge about the complex structure and biology of these phosphatases in hematologic cells, thereby providing the rationale behind their diverse signaling functions. Eventually, this basic knowledge is a key to truly understand the tumor suppressive role of PP2A in leukemogenesis and to allow further rational development of therapeutic strategies targeting PP2A.

Keywords: PP2A, subunit, inhibitor, tumor suppressor reactivation therapy, PP2A-activating drugs

INTRODUCTION

With 518 kinases encoded by the human genome and up to 70% of all eukaryotic proteins undergoing phosphorylation on a Ser, Thr, or Tyr residue, nearly every cellular process is controlled by this key modification (1). The covalent attachment of the bulky, negatively charged phosphoryl moiety to a protein markedly affects protein function through conformational changes that alter catalytic activity (for enzymes), affinity for ligands, subcellular localization, or stability (2). Several decades of biochemical and genetic studies have revealed crucial roles for protein kinases in the processes leading to tumor cell proliferation, survival, and migration in hematologic and other malignancies. In particular, genetic alterations that lead to constitutive activation of kinases, uncoupled from extracellular regulatory inputs, are well-characterized drivers of cancer development, a knowledge, which has emerged in the development of small-molecule kinase inhibitors for anti-cancer therapy (3). Kinase inhibitors have been extremely successful in the treatment of cancers driven by a single oncogenic kinase, such as chronic myeloid leukemia (CML) (4), but several challenges remain, including the development of drug resistance, lack of inhibitor selectivity or efficacy, and difficulty in drug target validation, particularly in cancers that do not exhibit such oncogenic kinase addiction (5).

Obviously, because protein phosphatases antagonize the action of protein kinases, they should be considered as equally important players in maintaining the correct phosphorylation balance of a given protein. Nonetheless, persistent misconceptions regarding the supposed lack of specificity and regulation of protein phosphatases as opposed to protein kinases, have contributed to a general underestimation of their critical role in the regulation of signal transduction (6,7). Hence, much less is known about their role in cancer development and progression. Research over the past decade has begun to highlight the importance of the tumor suppressive activities of protein phosphatases, which, upon functional inactivation, contribute to persistent kinase or oncogene activation, and perhaps even more importantly, to drug resistance development (8,9). Therefore, protein phosphatases may represent valuable novel drug targets for alternative cancer therapies, either in their own right or as part of combination therapies with kinase inhibitors (10–13).

Protein phosphatase 2A (PP2A) represents the prototype of a highly regulated phosphatase family with suspected critical tumor suppressive properties in several human tissues (14–16). Recent reports have demonstrated that modulation of PP2A activity can be beneficial for the treatment of cancer, particularly of hematologic malignancies (17,18). Increasing evidence from cellular and clinical studies has indeed underscored the tumor suppressive role of PP2A in leukemogenesis, although the complex biology of these enzymes in hematologic cells remains incompletely understood. Here, we will provide insights into the basics of PP2A structure and regulation in hematologic cells and tissues, and highlight how proper PP2A function or activity is affected in hematologic malignancies. This knowledge is not only imperative to understand the protective role of PP2A in leukemogenesis but also equally important to allow for

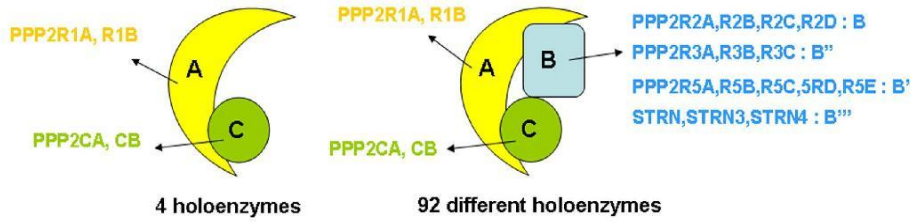

FIGURE 1 | Structure of PP2A holoenzymes. The majority of PP2A enzymes have a heterotrimeric structure and consist of one catalytic C subunit, one scaffolding A subunit, and one regulatory B-type subunit. Owing to the existence of various isoforms of each of these subunits – in human tissues, two C (encoded by *PPP2CA* and *PPP2CB*), two A (encoded by *PPP2R1A and PPP2R1B*), and 23 B-type isoforms (encoded by 15 different genes) – 92 different PP2A trimeric complexes can be assembled, each characterized by its own catalytic properties, substrate specificities, tissue or cell-specific expression, and subcellular localization. In addition, about one-third of PP2A occurs as a dimer of one A and one C subunit (four holoenzymes).

rational design of PP2A-directed drugs, and thus, to fully exploit PP2A as anti-cancer target in these devastating diseases.

PP2A FAMILY

PP2A ENZYMES: STRUCTURAL AND FUNCTIONAL CENTIPEDES

"PP2A" refers to a large, highly conserved family of ubiquitously expressed Ser/Thr phosphatases that, together with PP1, constitutes the bulk of Ser/Thr phosphatase activity in a given cell or tissue (19). The prototypic PP2A holoenzyme is a heterotrimeric complex of a catalytic C subunit, a scaffolding A subunit, and a regulatory B-type subunit (**Figure 1**). In human cells, B-type subunits are encoded by 15 different genes, which give rise to 23 different isoforms through use of alternative gene promoters, alternative splicing events, or alternative translation (20). Based on sequence homology, they are divided into four distinct families, called B (or B55, or PR55, or by gene name: *PPP2R2*), B' (or B56, or PR61, or by gene name: *PPP2R5*), B''(or PR72, or by gene name: *PPP2*R3), and B''' (or the striatins, *STRN*) (**Figure 1**). The B-type subunits are true "regulatory" subunits, in the sense that they dictate substrate specificity of the associated PP2A C subunit and can directly modulate PP2A catalytic activity. They are often expressed in a cell- or tissue-specific way, and can be found at distinct subcellular locations (cytoplasm, nucleus, plasma membrane, mitochondria, Golgi apparatus, endoplasmic reticulum, and cytoskeleton), thus, restricting PP2A activity to cell- or tissue-specific substrates present at specific subcellular sites (20,21). Also, the C and A subunits are encoded by two different genes each, giving rise to two nearly identical Cα and Cβ isoforms (encoded by *PPP2CA* and *PPP2CB*), and two highly related Aα and Aβ isoforms (encoded by *PPP2R1A* and *PPP2R1B*). Despite an extremely high degree of sequence identity, there is evidence that these isoforms, remarkably, do not serve redundant functions (22–25). Besides their assembly into trimeric PP2A complexes, A and C subunits can form active A–C heterodimers (**Figure 1**), which are estimated to represent about one-third of cellular PP2A in a given cell (26).

The combinatorial assembly of one C and one A, or one C, one A, and one B-type subunit can theoretically give rise to 4 different heterodimers and 92 different heterotrimers (**Figure 1**), all exhibiting potentially different physiological functions. Thus, the broad diversity in PP2A composition creates specificity and constitutes the basis for the highly diverse and multiple cellular and physiological functions of these phosphatases. PP2A has indeed been implicated in a wide range of signaling pathways, many of which are involved in the control of cell proliferation and death (16,27), cell division (28,29), differentiation (28), adhesion and migration (30), and metabolism (31,32). Besides function, PP2A composition also largely defines regulation by upstream factors, including specific second messengers (cAMP, Ca^{2+}-ions, lipids) (20), cellular PP2A inhibitors (33) (see further), and phosphorylation by specific kinases (20). Most of these regulatory inputs are again largely determined by the nature of the specific B-type subunit present in the complex.

This being said, it should come of little surprise that "PP2A" (i.e., the large family of distinct PP2A complexes) may exert collaborating as well as opposing functions within a given signaling pathway by acting at different levels in the cascade. This is, for instance, the case in growth factor-induced ERK signaling, TGFβ signaling, or in canonical and non-canonical Wnt signaling (16,27,34). In addition, different PP2A complexes may dephosphorylate the same substrate, even on the same site, depending on the regulatory stimulus involved, the cell type or the broader physiological context (35–37). In contrast, functional redundancies, particularly between PP2A complexes harboring a B-type subunit from the same subfamily, have also been reported (38), further illustrating the complexity of PP2A holoenzyme function and substrate selection. It is clear though that in order to fully understand the role and regulation of "PP2A" in any (patho)physiological context, it is of utmost importance to identify which particular holoenzymes are involved in a non-redundant way. Nonetheless, and despite their general importance in PP2A biology, the specific PP2A regulatory subunits controlling dephosphorylation of a given substrate in a given mammalian cell or tissue remain poorly defined, particularly in the physiological context of a whole organ-ism (39–41). Additional "PP2A" knockout mice are eagerly being awaited to overcome this lack of *in vivo* knowledge, and eventually, to advance the rational development of PP2A as a druggable target in the relevant cancer types.

EXPRESSION OF PP2A SUBUNITS IN SPLEEN, THYMUS, AND BONE MARROW

To truly understand the biology of "PP2A" in hematologic cells and tissues, one should ideally know which PP2A complexes occur in

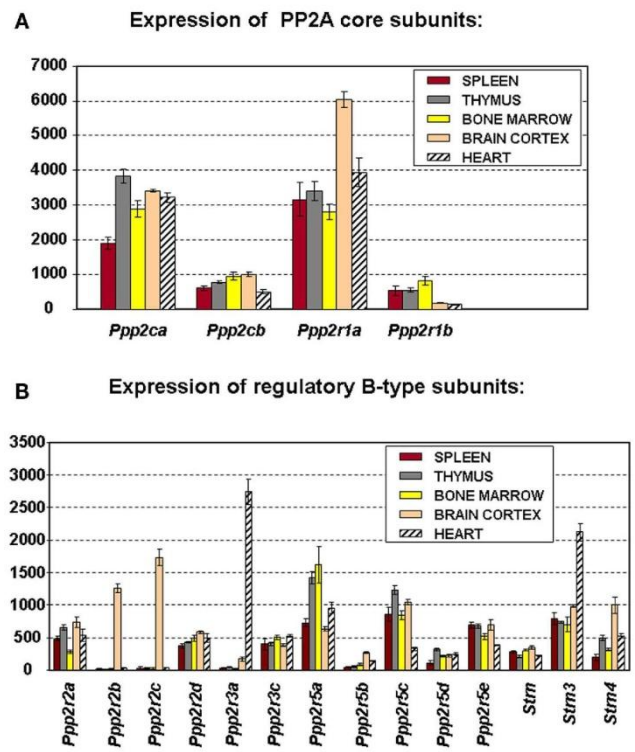

FIGURE 2 | Microarray expression profiles of PP2A subunit encoding genes in mouse tissues. Spleen, thymus, bone marrow, brain cortex, and heart were hand-dissected from 10- to 12-week-old C57Bl6 mice. Total RNA was extracted, labeled, and hybridized to the Affymetrix mouse MOE 430 2.0 array (44). Scanning, quality control, data processing, and statistical analysis of the data were as described (44). Shown is the mean mRNA expression signal SD of three (spleen, thymus, brain, and heart) or four (bone marrow) biological replicate experiments. **(A)** Expression of the PP2A core subunit encoding genes. **(B)** Expression of the genes encoding PP2A regulatory B-type subunits. Expression of *Ppp2r3d* could not be analyzed because it was not present on the array used.

these tissues. Because of general lack of sufficient isoform-specific antibodies and because only fragmented relevant information can be found in the currently available PP2A literature, we have analyzed, for the purpose of this review, mRNA expression of all PP2A subunit genes via microarray in mouse spleen (*n*=3), thymus (*n*=3), and bone marrow (*n*=4) (**Figure 2**). Brain cortex (*n*=3) and heart (*n*=3) were included as "controls" (**Figure 2**) as in these tissues, expression of most PP2A subunits has been investigated and reported before. If we presume that hybridization of the PP2A mRNAs to their respective gene probes on the chip occurs with comparable efficiency, we find overall significantly higher expression of the α isoforms of both C and A subunits as opposed to their respective β isoforms (**Figure 2A**), fully in accordance with published data (19,24). When considering expression of B-type subunits, most of them are expressed in all three hematologic tissues, except *Ppp2r2b* and *Ppp2r2c* (encoding Bβ and Bγ), which were reported to be exclusively expressed in brain (42), *Ppp2r3a* (encoding B''α), which was reported to be predominantly expressed in heart (43), and *Ppp2r5b* (encoding B'β) whose hematologic expression is extremely low (**Figure 2B**). Highest expression is seen for *Ppp2r5a* and *Ppp2r5c* (encoding B'α and B'γ), followed by *Ppp2r5e* (encoding B'ε), *Strn3* (encoding B'''/SG2NA), *Ppp2r3c* (encoding B''γ/G5PR), *Ppp2r2a*, and *Ppp2r2d* (encoding Bα and Bδ). Lowest expression is seen for *Ppp2r5d* (encoding B'δ), *Strn*, and *Strn4* (encoding B'''/striatin and B'''/zinedin) (**Figure 2B**). Expression of *Ppp2r3d* could not be analyzed because it was not represented on the microarray chip. For most PP2A subunits present in these tissues, expression is comparable between spleen, thymus, and bone marrow, except for *Ppp2r2a* (Bα), which is approximately two times more abundant in spleen and thymus as opposed to bone marrow, and for *Ppp2r5a* (B'α), *Ppp2r5d* (B'δ), and *Strn4* (B'''/zinedin), which are least abundant in spleen as opposed to thymus and bone marrow (**Figure 2B**). Thus, these data illustrate in a qualitative and semi-quantitative way the repertoire of PP2A B-type subunits expressed in the three main hematologic tissues in mice.

INACTIVE PP2A COMPLEXES AND PP2A HOLOENZYME ASSEMBLY

Besides the prototypical PP2A holoenzymes described above, several "atypical" PP2A complexes have been identified that can occur within cells as catalytically inactive PP2A complexes. For example, the interaction between the C subunit and the a4 protein (encoded by *IGBP1*) stabilizes the C subunit as a latent, inactive form (45,46), although there is also some evidence that this complex might be active toward very specific cellular substrates [reviewed in Ref. (47)]. Another example is the catalytically inactive complex between the C subunit, the A subunit, and PME-1 (PP2A Methyl Esterase 1, encoded by *PPME1*) (**Figure 3A**) that has been estimated to represent up to 25% of the cellular PP2A C pool (48,49). It is thought that these atypical, inactive PP2A complexes constitute intermediate, but stable complexes during the process of PP2A holoenzyme biogenesis (47) or holoenzyme disassembly (45). Interestingly, increased expression of α4 or PME-1 has been found in several human cancers [hepatocellular carcinoma (50), lung carcinoma (50, 51), breast cancer (50), glioma (52), and endometrial cancer (53)], indicative for a relative increase in inactive PP2A complexes as opposed to active holoenzymes in these transformed cells.

The precise mechanism of assembly of active PP2A holoenzyme is still incompletely understood (47). A major insight came from the finding that the PP2A C subunit is synthesized/translated as an inactive enzyme (54) that is subsequently activated in a way that is strictly coupled to its incorporation into the complete holoenzyme (55). Like that, promiscuous and unregulated phosphatase activity of the free C subunit can be avoided (54,55). There is evidence that proteins such as α4 and PME-1 can stabilize such inactive PP2A C subunits within cells, either in the absence (for α4) (46) or the presence (for PME-1) of the A subunit (47). To generate active PP2A holoenzymes from these inactive complexes, at least two additional PP2A regulating enzymes are needed. First, PTPA (or "PP2A Activator," encoded by *PPP2R4*) may activate the PME-1-bound PP2A complex in the presence of ATP/Mg^{2+} as necessary cofactors (**Figure 3A**) (49). In accordance, *in vivo* data in yeast have shown that PTPA-dependent generation of active C subunit requires a functional interaction with the A subunit and is regulated by PME-1 (55). Crystallographic data suggested that PTPA may act as an

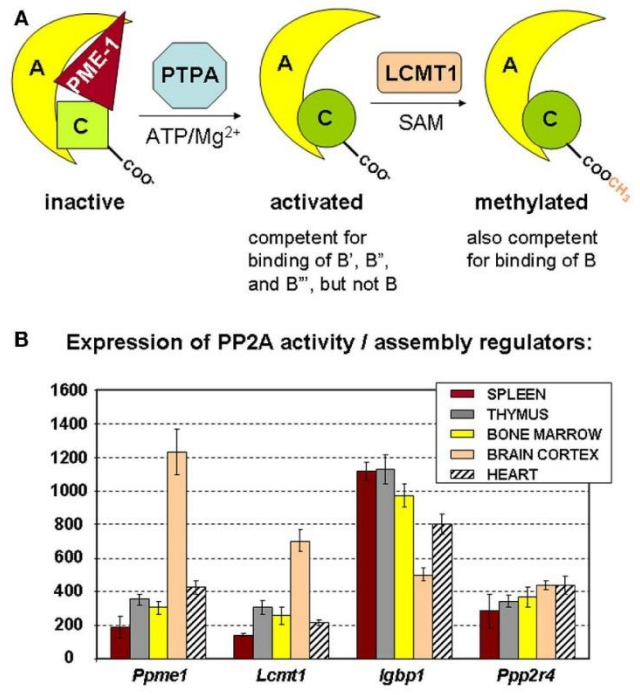

FIGURE 3 | Regulators of PP2A holoenzyme biogenesis and assembly. **(A)** Simplified schematic of the roles of PME-1, PTPA, and LCMT1 in the biogenesis of active PP2A trimers. The PP2A methylesterase PME-1 serves to stabilize the inactive PP2A C subunit in a complex with the A subunit, at the same time preventing PP2A C methylation. With ATP/Mg^{2+} as necessary cofactors, PTPA promotes folding of PP2A C in an active conformation, and thereby, indirectly, PP2A C carboxymethylation by LCMT1. The latter modification is absolutely required for binding of B subunits, facilitates interaction of all B' subunits but the δ isoform, is of no apparent importance for binding of B'δ and the B'' subunits, and is disliked by the striatin subunits. α4 (not depicted here) is another regulator that stabilizes PP2A C in a latent form. It is currently unclear if and how this inactive α4–C complex might become activated by similar mechanisms (47).
(B) Expression of PP2A biogenesis regulators in hematologic tissues, brain, and heart. The mean mRNA expression signal ± SD for *Ppme1* (PME-1), *Lcmt1* (LCMT1), *Igbp1* (α 4), and *Ppp2r4* (PTPA) is shown of three (spleen, thymus, brain, and heart) or four (bone marrow) biological replicate experiments.

ATP/Mg^{2+}-dependent prolyl-peptidyl *cis/trans* isomerase of a single prolyl-peptidyl bond in PP2A C (56), as well as an ATP/Mg^{2+}-dependent chaperone promoting the incorporation of catalytic metal ions into the PP2A C active site (57). Regardless of its precise mechanism-of-action, several *in vivo* studies have underscored the importance of PTPA as a physiological activator of PP2A [reviewed in Ref. (47)]. The second enzyme important in PP2A biogenesis is LCMT1 (leucine carboxyl methyl transferase 1, encoded by *LCMT1*), an *S*-adenosylmethionine-dependent methyltransferase catalyzing the carboxymethylation of the PP2A C subunit (58). This unusual post-translational modification of PP2A C is reversible through the presence of PME-1, the PP2A methylesterase (59), which may thus serve a dual function. Interestingly, PP2A C carboxymethylation requires an active PP2A C conformation (60), is facilitated by the presence of the A subunit (61), and enhances the affinity of the core dimer for PP2A regulatory subunits (**Figure 3A**). Specifically, PP2A C methylation is an absolute prerequisite to bind subunits of the B family, it facilitates interaction of all members but the δ isoform of the B' family,is of no apparent importance for binding of B' and the B'' subunits, and is disliked by the B''' subunits [reviewed in Ref. (62)]. Intriguingly, all regulators involved in PP2A holoenzyme biogenesis (α4, PME-1, PTPA, and LCMT1) are indispensable for mammalian survival (63– 65, unpublished work), indicative for their physiological importance. In accordance, they are all expressed in spleen, thymus, and bone marrow, the three hematologic tissues analyzed here by microarray (**Figure 3B**). Our data also show a relatively higher expression of PME-1 and LCMT1 in brain, as opposed to other tissues analyzed (**Figure 3B**).

CELLULAR PP2A INHIBITORY PROTEINS

Although the first cellular PP2A inhibitors were discovered almost two decades ago (66), their role in direct regulation of PP2A activity has only during the recent years come into considerable focus, not the least because some of them commonly suppress PP2A activity in hematologic and other cancers, and thus, may constitute novel therapeutic targets (33). These inhibitors either directly bind to the PP2A catalytic subunit or target very specific PP2A holoenzymes, thereby preventing dephosphorylation of a large variety of PP2A substrates (**Figure 4A**).

ANP32a AND SET

PP2A inhibitor 1 (also called ANP32a, or by gene name: *ANP32a*) and inhibitor 2 (also called TAF-Iβ, or PHAP1, or by gene name: *SET*) were originally *de novo* purified from bovine kidney as two potent, heat-stable PP2A-specific inhibitors (66) and subsequently cloned from cDNA libraries (67,68). ANP32a belongs to a large family of at least nine members (ANP32a–h), of which only ANP32a and ANP32e show PP2A inhibitory ability in an *in vitro* phosphatase assay (69,70). Free PP2A C subunit, the core A–C dimer and a trimeric PP2A complex with B subunit were inhibited in this assay, suggestive for direct binding of ANP32a to the C subunit, and thus, for no specific holoenzyme selectivity. Essentially, the same observations were made for SET in *in vitro* PP2A phosphatase assays (68). SET exists as two splice variants (SETα and β) that are both capable of inhibiting PP2A (71). ANP32a/e and SET are all phosphoproteins and can be found in the nucleus as well as the cytoplasm. Tyrosine phosphorylation of ANP32a releases its binding to PP2A and relieves PP2A inhibition toward MEK (72). In SETα, Ser9, Ser24, Ser93, and Ser171 have been identified as phosphorylation sites of functional importance (73–75). Phosphorylation of Ser9 functionally disrupts a nuclear localization signal and promotes SET retention in the cytoplasm (76,77), while Ser171 phosphorylation decreases its PP2A inhibitory potential (74) and Ser9/Ser93 phosphorylation increases its ability to inhibit PP2A (75). Proteolytic cleavage of SET, either by Granzyme A at K176 (78) or by asparaginyl endopeptidase at N175 (79) is another mechanism that promotes its translocation into the cytoplasm (80), while the SET-binding protein SETBP1 stabilizes full-length SET inside the nucleus (81). Several PP2A substrates are known to be affected by SET, either directly or indirectly, including ERK1/2 (82), Akt (82–84), PTEN (83), Mcl-1 (85,86), c-Myc

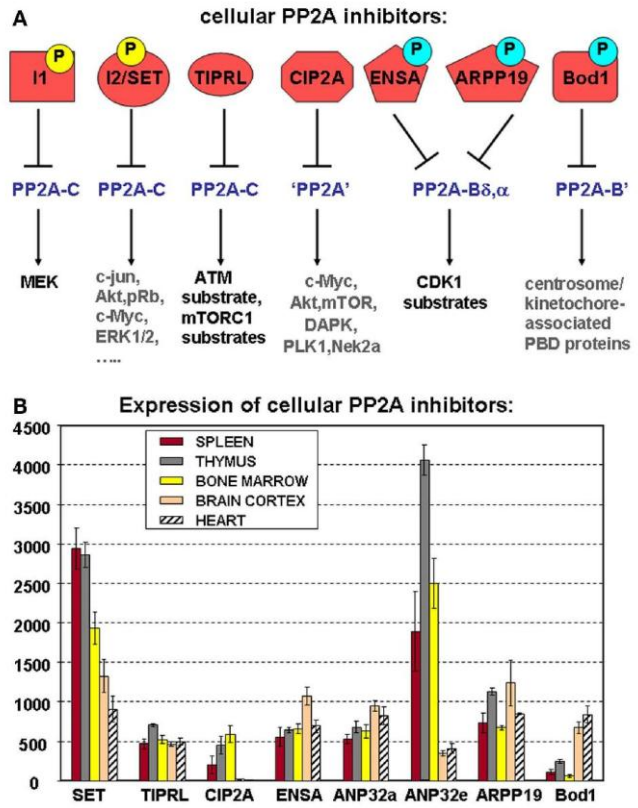

FIGURE 4 | Cellular PP2A inhibitors. **(A)** Schematic representation of known cellular PP2A inhibitors, highlighting their potential regulation by (yellow) or dependence on (blue) phosphorylation, their holoenzyme specificity (if known), and the PP2A substrates they affect. Best characterized so far, in terms of phosphorylation dependence and holoenzyme specificity, are the mitotic inhibitors ENSA, ARPP-19, and Bod1. Both I1 (ANP32a, e) and I2 (SET) are established phosphoproteins, but depending on the specific site of modification, these phosphorylations may increase as well as decrease their PP2A inhibitory abilities. Phosphoregulation of CIP2A or TIPRL has not yet been described. I1, I2, and TIPRL are thought to interact with PP2A complexes through the C subunit, while holoenzyme specificity of CIP2A-mediated inhibition remains undefined. PBD: polo-box domain. **(B)** Expression of cellular PP2A inhibitors in hematologic tissues, brain, and heart. The mean mRNA expression signal ± SD for the eight indicated cellular PP2A inhibitory proteins is shown of three (spleen, thymus, brain, and heart) or four (bone marrow) biological replicate experiments.

(82,84,87), c-jun (88),and pRb (82), just to name a few (**Figure 4A**).

CIP2A

CIP2A (or cancerous inhibitor of PP2A, or by gene name *KIAA1524*) is an oncoprotein, originally identified as a novel co-precipitating partner of the PP2A A subunit (89). CIP2A is barely detectable in normal cells, but becomes specifically upregulated in a large variety of human cancers, hence its name [reviewed in Ref. (90)]. CIP2A knockout mice show no overt phenotypes, except for a defect in spermatogenesis (91). In cancer cells, CIP2A upregulation is mediated by several oncogenic transcription factors, including Ets (92), Myc (93), and E2F (94), and often correlates with cancer aggressiveness and poor prognosis (90). At the signaling level, increased CIP2A expression has been associated with increased c-Myc stability and Ser62 phosphorylation (89), increased Akt signaling (95), inhibition of dependence receptor-dependent apoptosis (96), and more recently, with changes in regulation of cell cycle kinases such as Plk1 (97) and NEK2 (98), and activation of the TOR pathway (99,100) (**Figure 4A**). The biochemistry of CIP2A remains, however, largely undefined; in particular, it remains to be determined which PP2A complexes it may specifically inhibit and how this is achieved.

TIPRL1

TIPRL1 (also called TIP, or two A inhibitory protein, gene name *TIPRL*) is a ubiquitously expressed PP2A inhibitory protein that has been shown to inhibit free PP2A C and the PP2A A–C dimer by an allosteric mechanism (101,102). TIPRL1 directly interacts with PP2A C, as well as with the C subunits of the PP2A-like phosphatases PP4 and PP6 (103). Notably, TIPRL1 may play an important role in DNA damage and repair signaling as it regulates PP2A enzymes that oppose ATM/ATR-dependent phosphorylation events (101). In addition, it may facilitate mTORC1 signaling and increase protein translation by sustaining phosphorylation of the mTORC1 substrates S6K1 and 4E-BP1 (104). In cancer cells, highly elevated TIPRL1 expression was reported in hepatocellular carcinoma, correlating with decreased pro-apoptotic MKK7/JNK signaling and contributing to resistance to TRAIL-induced apoptosis (105). The physiological role of TIPRL1 remains, however, undefined.

MITOTIC PP2A INHIBITORS: ENSA, ARPP-19, AND Bod1

cAMP-regulated phosphoproteins ARPP-16 and ARPP-19 are splice variants and members of an evolutionary conserved protein family, to which ENSA (α-endosulfine) is closely related. ENSA and ARPP-19 are mitotic PP2A inhibitors that promote the G2/M transition and the mitotic state (106,107). Intriguingly, they strongly bind to Bα and Bδ, but no other B-type subunits, dimeric PP2A or monomeric PP2A C, suggestive for a strong PP2A holoenzyme specificity (106–108) (**Figure 4A**). Moreover, these proteins require prior phosphorylation by the mitotic Great-wall/MASTL kinase, the Cdk1/cyclinB kinase, or potentially other mitotic kinases to exert their PP2A inhibitory effects (106,107,109,110). Phosphorylation of ARPP-19 by cAMP-dependent kinase (PKA) serves to keep oocytes arrested in prophase (111), but how this may affect PP2A inhibition is unknown. More recently, yet another mitotic PP2A inhibitor was identified: Bod1, a protein required for proper chromosome alignment at mitosis. Bod1 shares sequence similarity with ENSA and ARPP-19, but intriguingly, specifically inhibits kinetochore- and centrosome-associated PP2A-B' holoenzymes. Again, a phosphorylation of Bod1 by Cdk1/cyclinB is required to promote interaction with and inhibition of PP2A-B' (112). Although many more needs to be discovered about the biochemistry and physiological roles of these mitotic PP2A inhibitors, they currently represent an exemplary mechanism of holoenzyme (family)-specific PP2A inhibition.

EXPRESSION OF PP2A INHIBITORS IN HEMATOLOGIC TISSUES

As for the PP2A subunits (**Figure 2**) and the regulators of PP2A holoenzyme assembly (**Figure 3B**), we have analyzed mRNA expression of the above PP2A inhibitors in mouse spleen (*n*=3), thymus (*n*=3), and bone marrow (*n*=4) (**Figure 4B**). Expression of

SET, CIP2A, and ANP32e appears significantly higher in all three hematologic tissues analyzed, as opposed to terminally differentiated brain and heart tissues. In fact, CIP2A expression is completely undetectable in brain and heart, consistent with the idea that its expression is tightly coupled to cell proliferation, and potentially, stemness (90). Bod1 expression shows the opposite behavior and is significantly less expressed in spleen, thymus, and bone marrow as opposed to brain and heart, while TIPRL1, ANP32a, ENSA, and ARPP-19 expression is comparable in tissues analyzed (**Figure 4B**).

PP2A ABERRATIONS IN HEMATOLOGIC MALIGNANCIES

Several mechanisms of PP2A dysfunction in hematologic malignancies have been reported, including changes in expression of PP2A subunits and inhibitors (by epigenetic or other mechanisms), genomic alterations in PP2A subunit and regulator encoding genes (including mutations, deletions, splicing errors, chromosomal translocations), and alterations in subunit modifications affecting PP2A activity.

ALTERATIONS IN PP2A SUBUNITS

Although both *PPP2R1A* (Aα) and *PPP2R1B* (Aβ) have been identified as genuine tumor suppressor genes in solid cancers (17, 25), few reports have currently documented their inactivation in hematologic malignancies. Decreased A subunit expression is observed in myeloid cells expressing activated c-KIT mutants (113), while loss of Aβ function occurs with low frequency in ALL (G90D mutation, 3/150) (114), B-CLL (exon skipping and reduced mRNA expression) (115,116), and AML (117). Decreased expression of Cα is one of the hallmarks of del(5q) myelodysplastic syndromes (MDS) and AML, and interestingly, predicts a favorable therapy response to lenalidomide (118), suggestive for its use as a stratification marker. Increased Y307 phosphorylation of PP2A C occurred in 29/37 AML cases, correlating with significantly decreased PP2A activity toward Akt and ERK (117).

PP2A B-type subunit alterations occur more frequently, particularly in AML. Reduced expression of Bα in AML blasts, correlating with increased Akt, p70S6K, and PKCα phosphorylation and deregulated expression of specific microRNAs (miRs), is associated with significantly reduced complete remission duration (119,120). In c-KIT mutant AML, reduced expression of Bα is observed, along with decreased expression of several B' subunits (α,γ,δ), correlating with overall decreased PP2A activity (113). Genomic deletion of *PPP2R5B/C* (B'β,γ) (117) and downregulation of B'ε by an elusive non-genomic mechanism (121) do also frequently occur in AML, correlating with increased oncogenicity of the leukemic cells. In lymphocytic leukemia, *PPP2R5C* (B'γ) downregulation is a hallmark of progressive as opposed to stable B-CLL (122), while in Notch-induced T-ALL, *PPP2R5E* (B'ε) was identified as one of the targets for miR-19, an oncomiR that promotes leukemogenesis *in vivo* (123). In childhood T-ALL and B-ALL, *PPP2R3A* (B''α) is epigenetically inactivated by increased methylation with high frequency (69 and 82%, respectively) (124). Genomic deletion of *PPP2R2A/B* (Bα,β) is sporadically observed in primary plasma cell leukemia and multiple myeloma (125,126).

ALTERATIONS IN PP2A REGULATORS

The large majority of PP2A aberrations in hematologic malignancies involve abnormalities (overexpression, genetic modifications) in the proto-oncogenic PP2A inhibitors CIP2A and SET.

The first time deregulated CIP2A expression was linked to blood cancer development was through the discovery of a chromosomal translocation, resulting in an MLL-KIAA1524 fusion protein in an isolated case of infant AML (127). In this fusion, exons 1–10 of MLL are coupled in frame to exons 17–21 of CIP2A, encompassing the CIP2A coiled coil domain. In addition, CIP2A overexpression occurs frequently in newly diagnosed AML (54/70) and relapsed AML (11/14) (128). In CML, a positive feedback loop between CIP2A and BCR/ABL has been described, implying that CIP2A overexpression may promote CML pathogenesis (129,130). Importantly, and in contrast to expression of SET, CIP2A expression is a clear determinant of disease progression to blast crisis (129) and thus confers a poor prognosis in these patients. Mechanistically, high-CIP2A levels in primary CML correlate with high levels of S62-phosphorylated c-Myc (129) and increased resistance to bortezomib-induced apoptosis (131). Analysis of CIP2A expression levels in a panel of 105 B-cell lymphomas further demonstrated a link with clinical aggressiveness of the subtypes, with weak or absent CIP2A expression in indolent B-cell lymphomas and strongly positive signals in the more aggressive diffuse large B-cell and Burkitt lymphoma subtypes (132).

Increased SET expression is found in CML, where it correlates with blast crisis and resistance to therapeutic BCR/ABL tyrosine kinase inhibitors (TKI) (82), in Philadelphia chromosome-positive (Ph)-ALL (133), (c-KIT positive) AML (113,134), and B-CLL (85). In AML and B-CLL, its expression is associated with disease severity and poor outcome. In leukemic progenitors, PP2A activity is substantially impaired as a result of SET overexpression (82). In CML and Ph-ALL, induction of SET expression is controlled by BCR/ABL (82), while in AML, overexpression of EVI1 or downregulation of miR199b may contribute (134). Restoration of PP2A activity in leukemic cells results in decreased phosphorylation of pRb, c-Myc, Stat5, ERK1/2, Akt, Bad, and Jak2, and induction of SHP1-mediated BCR/ABL inactivation and degradation (82,135). In atypical CML, lacking the BCR/ABL fusion, recurrent *SETBP1* mutations are found in 17/70 cases, some of which abrogate a site for ubiquitination, resulting in increased amounts of SETBP1 and SET protein, lower PP2A activity, and higher proliferation rates (136). In AML, overexpression of SETBP1 predicts poor outcome in elderly AML patients (81). Finally, *SET* is recurrently involved in chromosomal rearrangements and translocations, in particular, with the nucleoporin-encoding *Nup214* gene (also called *CAN*) in AML, T-ALL, and acute undifferentiated leukemia (137–140).

PP2A (RE)ACTIVATION AS A NOVEL THERAPEUTIC STRATEGY IN HEMATOLOGIC MALIGNANCIES

The above findings, highlighting several mechanisms of PP2A inactivation in patients with hematologic malignancies, substantially underscore the tumor suppressor activities of (specific) PP2A holoenzymes and the proto-oncogenic properties of PP2A inhibitors CIP2A and SET. Importantly, some of these mechanisms may serve as biomarkers to improve current therapies (118), or may be directly amenable for therapeutic intervention. Several recent preclinical

studies have shown that pharmacological restoration of PP2A tumor suppressor activity by PP2A-activating drugs (PADs) indeed effectively antagonizes cancer development and progression [reviewed in Ref. (17)]. Because PP2A complexes have so many cellular targets, these therapies may have the additional advantage, not to target just a single oncogene, but rather many different oncogenic pathways, contributing to their therapeutic efficacy. On the other hand, it is obvious that not all mechanisms of PP2A inhibition are suitable for restoration, particularly when subunit mutations are involved. Likewise, the development of small-molecule protein–protein interaction inhibitors targeting PP2A–SET or PP2A–CIP2A complexes remains, although attractive, extremely challenging.

Neviani et al. were the first to highlight the therapeutic relevance of using PP2A activators, such as FTY720 and forskolin, to target leukemia cells (82,133). These observations prompted many others to test these compounds successfully in their own leukemic models (85,113,117,141,142). Treatment of AML patients with forskolin, in combination with standard induction therapy, gave an additive effect, highlighting therapeutic potential of PP2A activators in combination with standard chemotherapy (117). The mechanism of PP2A activation by these compounds remains somewhat obscure, but may involve direct binding of FTY720 to a ceramide-binding domain of SET (143,144), resulting in SET dissociation from PP2A (143,145). FTY720 also reduces SET Ser phosphorylation (144) and promotes SET nuclear localization (145), suggesting that its therapeutic effect may be largely attributable to restoration of cytoplasmic PP2A activity. The cell penetrating SET antagonistic peptides COG112 and OP449 (formerly COG449) directly bind SET to prevent SET–PP2A inter-action and enhance PP2A activity (84). Like FTY720, they show significant therapeutic potential as PADs, as they induce apoptosis of human B-cell non-Hodgkin lymphoma and B-CLL *in vitro* and *in vivo*, without any discernable effects on normal B cells (85,146). In models of human CML and AML (147) and canine T-cell lymphomas (148), OP449 also shows anti-tumoral effects, especially in combination with ABL TKI (147). The latter demonstrates the added benefit of combining TKIs and PADs for anti-leukemic therapy (17). Very recently, yet another class of FDA-approved drugs, the phenothiazines, were shown to act as PADs in models of T-ALL (149). These compounds induce rapid dephosphorylation of multiple PP2A targets, resulting in suppressed growth and increased apoptosis of T-ALL cells *in vitro* and *in vivo*. Mechanistically, a direct interaction with the Aα subunit is involved, but how this results in increased PP2A activity should still be further explored.

Together, these findings strongly encourage the inclusion of pharmacological PP2A activators with major anti-cancer activities and good safety profiles into current anti-cancer protocols in hematologic malignancies. The partially overlapping effects of existing drugs and PP2A stimulation predict that the inclusion of PADs in combination therapies with TKIs or other conventional therapeutics would represent particularly attractive therapeutic strategies to improve therapeutic outcome in these devastating malignancies. In the meantime, additional efforts to improve the potency and selectivity of existing PADs, and to identify alternative PP2A-activating strategies should be undertaken, in order to achieve their eventual use in the clinic.

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5

Results (1):

B56δ-related protein phosphatase 2A dysfunction identified in patients with intellectual disability

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B56δ-related protein phosphatase 2A dysfunction identified in patients with intellectual disability

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Here we report inherited dysregulation of protein phosphatase activity as a cause of intellectual disability (ID). De novo missense mutations in 2 subunits of serine/threonine (Ser/Thr) protein phosphatase 2A (PP2A) were identified in 16 individuals with mild to severe ID, long-lasting hypotonia, epileptic susceptibility, frontal bossing, mild hypertelorism, and downslanting palpebral fissures. PP2A comprises catalytic (C), scaffolding (A), and regulatory (B) subunits that determine subcellular anchoring, substrate specificity, and physiological function. Ten patients had mutations within a highly conserved acidic loop of the *PPP2R5D*-encoded B56δ regulatory subunit, with the same E198K mutation present in 6 individuals. Five patients had mutations in the *PPP2R1A*-encoded scaffolding Aα subunit, with the same R182W mutation in 3 individuals. Some Aα cases presented with large ventricles, causing macrocephaly and hydrocephalus suspicion, and all cases exhibited partial or complete corpus callosum agenesis. Functional evaluation revealed that mutant A and B subunits were stable and uncoupled from phosphatase activity. Mutant B56δ was A and C binding–deficient, while mutant Aα subunits bound B56δ well but were unable to bind C or bound a catalytically impaired C, suggesting a dominant-negative effect where mutant subunits hinder dephosphorylation of B56δ-anchored substrates. Moreover, mutant subunit overexpression resulted in hyperphosphorylation of GSK3β, a B56δ-regulated substrate. This effect was in line with clinical observations, supporting a correlation between the ID degree and biochemical disturbance.

Introduction

Unlike protein kinases, mutations in serine/threonine (Ser/Thr) protein phosphatases have not commonly been associated with disorders of human development. There are 2 major Ser/Thr protein phosphatase families in the cell: protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), together accounting for more than 90% of all phospho-Ser/Thr dephosphorylations. PP2A consists of a catalytic subunit (C), a substrate binding regulatory subunit (B), and a scaffolding subunit (A) that links B and C. Unlike the generally expressed A and C subunits, there is a plethora of B subunits with different expression patterns (1). The differential substrate preferences of the nearly 100 different PP2A holoenzymes that, in theory, can be formed by 2 C isoforms, 2 A isoforms, and at least 23 types of B subunits is largely unknown (2), particularly within the context of a whole organism. Apparently, PP2A-dependent protein dephosphorylation has a potential for regulation that may be just as fine-tuned as protein phosphorylation. Unlike protein phosphorylation, associations between mutations in PP2A subunits and genetic diseases or syndromes have not been described until recently, when 4 de novo *PPP2R5D* and 3 de novo *PPP2R1A* mutations were found among the first 1,133 parent-child trios sequenced in the United Kingdom Deciphering Developmental Disorders project (3).

Here, we add clinical descriptions and functional data to the DDD findings and present 9 additional cases with de novo PP2A subunit mutations; 7 in *PPP2R5D*, encoding the regulatory B56δ subunit, and 2 in *PPP2R1A*, encoding the scaffolding Aα subunit.

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Table 1. De novo mutation details and the corresponding cases

Cases	Gene	Genomic change[A]	cDNA change[B]	Protein change
1	*PPP2R5D*	chr6:g.42974253C>T	c.157C>T	p.Pro53Ser
2–7	*PPP2R5D*	chr6:g.42975003G>A	c.592G>A	p.Glu198Lys
8–9	*PPP2R5D*	chr6:g.42975009G>A	c.598G>A	p.Glu200Lys
10	*PPP2R5D*	chr6:g.42975013C>G	c.602C>G	p.Pro201Arg
11	*PPP2R5D*	chr6:g.42975030T>A	c.619T>A	p.Trp207Arg
12	*PPP2R1A*	chr19:g.52715971C>T	c.536C>T	p.Pro179Leu
13–15	*PPP2R1A*	chr19:g.52715979C>T	c.544C>T	p.Arg182Trp
16	*PPP2R1A*	chr19:g.52716329G>A	c.773G>A	p.Arg258His

[A]Genomic positions are according to Build37/hg19. [B]cDNA reference sequences: NM_006245.2 for *PPP2R5D* and NM_014225.5 for *PPP2R1A*.

Taken together, of the 11 mutations in *PPP2R5D*, 6 mutations and 2 mutations were identical; 3 of the 5 mutations in *PPP2R1A* were also identical. All Aα mutations and all but one of the B56δ mutations had the potential to hinder access of catalytically competent C subunits to B56δ-regulated substrates, suggesting a common dominant-negative disease mechanism mainly affecting B56δ-regulated Ser/Thr dephosphorylation.

Results

In cases with intellectual disability (ID) of unknown etiology, parent-child trio exome sequencing was performed to find de novo and recessive mutations that could explain the condition. De novo missense mutations in 2 subunits of the Ser/Thr phosphatase PP2A were identified in 16 individuals from the United Kingdom (7 cases), the Netherlands (7 cases), Israel (1 case), and Norway (1 case).

The 7 United Kingdom cases were found among 1,133 chromosomally normal parent-child trios (3). This suggests that the prevalence of PP2A subunit mutations in the moderate-to-severe ID group without pathogenic copy number aberrations is around 0.6%. In the United Kingdom, this was part of the large DDD project (http://www.ddduk.org); in other cases, this was done as part of routine diagnostics. In 11 cases, de novo missense mutations in *PPP2R5D*, encoding the regulatory B56δ PP2A subunit, were found. In 5 other cases, a de novo missense mutation in *PPP2R1A*, encoding the scaffolding Aα subunit of PP2A, was found. Six mutations and 2 mutations in *PPP2R5D* were identical, and 3 *PPP2R1A* mutations were identical. Details on all mutations can be found in Table 1. Other trio exome sequencing results indicating a de novo change of possible relevance or a recessive condition of potential interest can be found in Supplemental Table 1 (supplemental material available online with this article; doi:10.1172/JCI79860DS1). In 10 cases, such findings were made, but based on bioinformatic evaluation of the variants and the clinical features of the patients, all but one of these findings could easily be excluded as causative factors for the phenotype. The exception was case 15, which had heterozygosity for a *TMEM67* splice mutation and a few signs that were compatible with a ciliopathy (e.g., unilateral postaxial polydactyly). However, this could also be a random finding, since a second *TMEM67* mutation was not found upon Sanger sequencing. In addition, detecting the same de novo missense mutations in patients with identical clinical features is, in itself, evidence in support of causality, especially when supported by functional data (see below). As a crude estimation, the likelihood of finding 10 de novo missense mutations in the same 9–amino acid stretch of B56δ by chance should be less than 10^{-50} (see Statistics).

The clinical features of the 11 *PPP2R5D* cases and the 5 *PPP2R1A* cases are summarized in Tables 2 and 3, respectively. Despite mutations occurring in 2 different PP2A subunit genes with different biochemical functions (regulatory and scaffolding), there are clinical similarities between the cases. All patients were born after a normal pregnancy, and 15/16 cases had birth weights within normal range. In 2 cases, breech deliveries were reported, and in 2 other cases, emergency cesarean sections had to be performed. After birth, ID and hypotonia were common features in all cases. Despite pronounced and long-lasting hypotonia, feeding difficulties were usually not a major problem, and only one case had gastrostomy. In 12/16 cases, the degree of ID was severe, and this correlated with very late independent walking, usually around age 6–7 years. The exceptions were the 4 patients with E200K, P201R, or W207R *PPP2R5D* mutations (see below for functional explanation), who learned to walk between 1½ and 2¼ years of age and had mild/moderate ID (Table 2). These 4 cases were also the only ones with language development beyond a few words. Seven out of 16 patients had epilepsy, including one of the mild ID cases. Only one patient had short stature (case 1 with a P53S mutation, see Table 2), and he was the only *PPP2R5D* case that was microcephalic. In the other *PPP2R5D* cases, head circumferences were from upper-normal range to pronounced macrocephaly, and in the latter cases, hydrocephalus was suspected. In contrast, most *PPP2R1A* cases were normocephalic or microcephalic, and hydrocephalus was initially suspected in only one case (Table 3). In all these patients, the corpus callosum was absent or almost absent, a feature that distinguished *PPP2R1A* cases from *PPP2R5D* cases. In contrast, facial features were overlapping (Figure 1): A hypotonic and sometimes also elongated face with tented upper lip, mild hypertelorism with downslanting palpebral fissures, and frontal bossing in the *PPP2R5D* cases.

The finding of recurrent and clustered de novo missense mutations in 2 PP2A subunit genes (*PPP2R5D* and *PPP2R1A*) suggested a dominant-negative– or gain-of-function–related disease mechanism, rather than haploinsufficiency or loss-of-function. All but one of the *PPP2R5D* mutations (E198K, E200K, P201R,

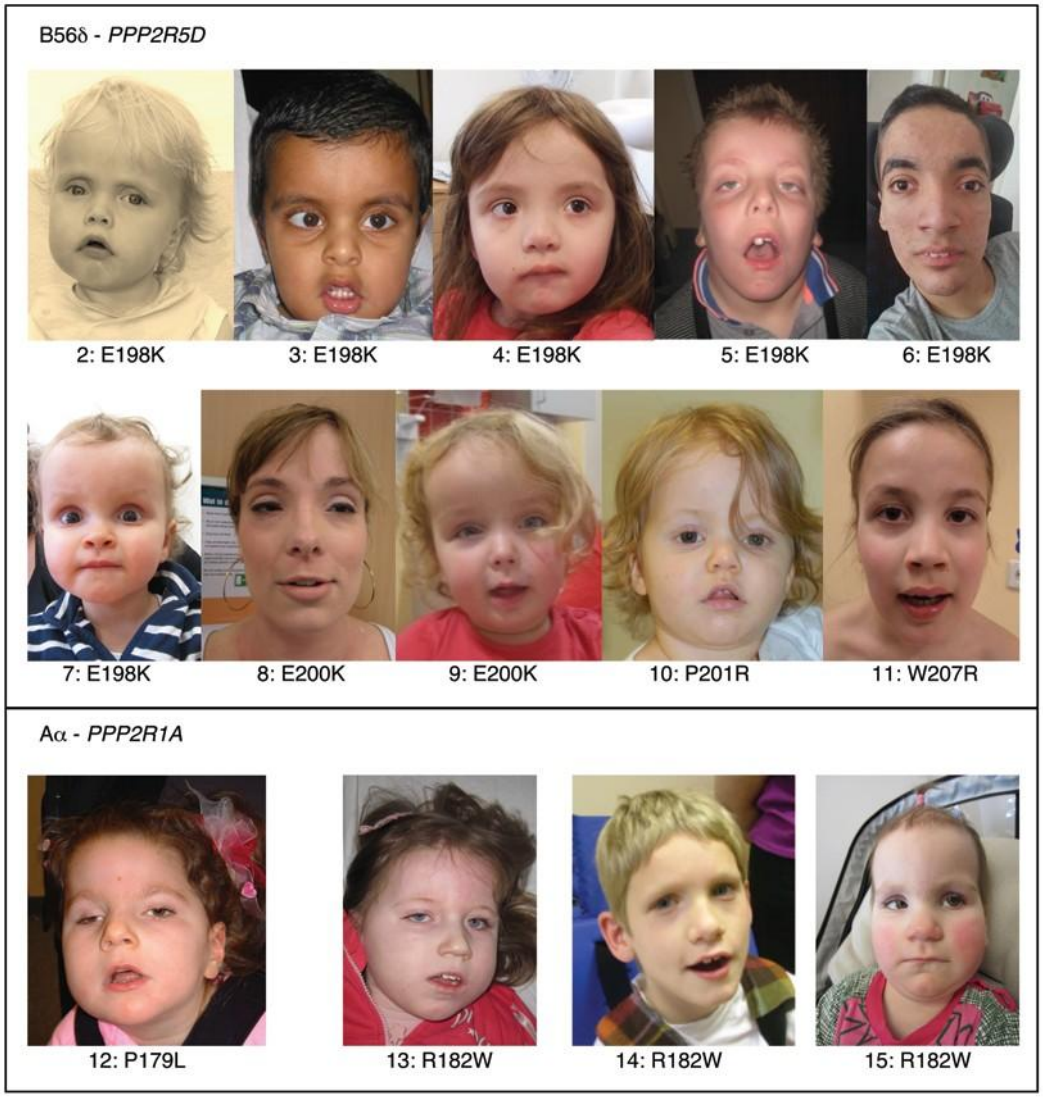

Figure 1. Facial photographs. The display shows the facial features of the *PPP2R5D* cases that consented to facial photographs being shown and the *PPP2R1A* cases.

and W207R) clustered in a highly conserved acidic loop that faces the A and C subunits (4–6). This acidic surface corresponds to the extended loop between α-helices 3 and 4 of HEAT domain 2 in the crystal structure of the highly related B56γ isoform (Figure 2A). Only the P53S mutation (case 1 in Table 2) localized outside this loop, i.e., in the B56δ-specific N-terminal domain.

To investigate if the *PPP2R5D* missense mutations affected subunit interactions, a human embryonic kidney cell line — HEK293 cells, a well-known model from previous PP2A subunit interaction studies (7) — was transfected with EGFP-tagged WT or mutant B56δ subunits in order to study subunit interactions. All ID-associated B56δ mutants except P53S showed deficient holoenzyme formation, i.e., A- or C-to-B56δ association (Figure 2B). To check if others had discovered missense variants in the same acidic B56δ loop, Broad institute's ExAC browser (http://exac.broadinstitute.org/) was consulted. Only 2 other missense variants (P196L and P201S) were reported. Interestingly, the latter variant was in the same residue as the de novo mutation (P201R) in case 10, although the amino acid change was different. Unlike P201R, we found that P201S failed to show any significant A or C binding defects (Supplemental Figure 1), further strengthening our working hypothesis that a charge change in the acidic B56δ loop could be pathogenic.

Our cellular binding assays with Glutathione S-Transferase–tagged (GST-tagged) B subunits and HemAgglutinin-tagged (HA-tagged) WT or mutant Aα subunits revealed that all 3 *PPP2R1A* mutations also affected PP2A holoenzyme formation (Figure 3). Surprisingly, interaction with the C subunit was hindered, despite the Aα mutations being in HEAT domains predicted to interact with B (Figure 3A). The mutations' effect on B subunit binding was complex (Figure 3B). All Aαmutants lacked significant binding to the B55 (also called B) family members tested (isoforms B55α and B55β), as well as to the B56 (also called B′ or PR61) family members tested (B56α and B56γ). On the other hand, binding to B56δ was almost entirely retained, whereas B56ε bound significantly less. For PR72, a member of the B′′ family of PP2A regulatory subunits, binding was retained to Aα-P179L but was completely lost to the Aα-R182W and Aα-R258H mutants (Figure 3B). These (mutant) Aα binding characteristics were confirmed for endogenous B55α and B56δ subunits, for which good-quality, isoform-specific antibodies are available (Figure 4A). These data could be compatible with a dominant-negative effect on, notably, B56δ for all Aα mutants, and on PR72

Table 2. Clinical features in cases with de novo *PPP2R5D* missense mutations

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8	Case 9	Case 10	Case 11
Mutation	P53S	E198K	E198K	E198K	E198K	E198K	E198K	E200K	E200K	P201R	W207R
Age of examination	53 yr	5 yr	11 yr	10 yr	15 yr	13 yr	2 yr	20 yr	4 yr	3 yr	9 yr
Sex	Male	Female	Male	Female	Male	Male	Female	Female	Female	Female	Female
Delivery	Normal	Breech	Normal	Emergency C/S	Emergency C/S	Normal	Amniotic rupture	C/S	Normal	Breech	Normal
Birth weight	Normal	Normal	Normal	Normal	Normal	SGA	Normal	Normal	Normal	Normal	Normal
Hypotonia	Not reported	Present	Present	Present	Present	Present	Present	Present	Present	Present	Present
Walked unsupported	9 yr	6 yr	6 yr	Not so far	7 yr	6 yr	Not so far	1½ yr	2¼ yr	1½ yr	2 yr
Ataxic gait	No	Yes	Yes	N/A	No	Yes	N/A	No	Yes	Yes	No
Language	No words	3–4 words	No words	2 words, poor articulation	No words	No words	No words	Yes, poor intelligibility	A few words	A few words	Yes, poor intelligibility
Epilepsy	No	No	No	No	Multifocal	Yes	No	No	No	Multifocal	No
EEG	–	Abnormal	–	Normal	Abnormal	Abnormal	Normal	Normal	Not done	Abnormal	Normal
ID/DD	Severe	Severe	Severe	Severe	Severe	Severe	Severe	Mild	Mild	Moderate	Moderate
Height	0.5 cm < 3rd	10th	5th	25th	20 cm < 3rd	50th	90th	5–10th	50th	50th	50th
Head circumference	1 cm < 3rd	5 cm > 97th	50th	5 cm > 97th at age 3½ yr	50th	97th	99th	97th	1 cm > 97th	75th	97th
Weight	Normal	Normal	50th	90th	50th	90th	30th	10 kg > 97th	75th	60th	50th
Brain MRI	–	Pseudo-hydroceph	Normal	Hydrocephalus	Mild ventricular dilatation	Mild ventricular dilatation Small CC	Normal	–	Normal	–	Normal
Other findings	Cataract			Fatigue Hypoglycemia Abnormal fat oxidation Bilateral 6th nerve palsies	Narrow palate Mild 2/3 and 3/4 finger syndactyly	Scoliosis	Neonatal nonepileptic myoclonus	Fatigue Ptosis	Fatigue Strabismus	Hip dysplasia Gastric reflux	Scoliosis Hip dysplasia Fatigue Mild mitochondrial dysfunction

Height, head circumference, and weight are measured relative to centiles (the 3rd and 97th centile correspond to ± 2 SD). Abbreviations: ID/DD, intellectual disability/developmental delay; y, years; -, unknown or not done; SGA, small for gestational age; C/S, cesarean section; CC, corpus callosum.

for Aα-P179L, provided that C binding would be lost or diminished in the B56δ-Aα mutant complex. To provide direct evidence that mutant Aα can complex with B56δ without C, we expressed HA-Aα mutants or HA-Aα (WT) in HEK293 cells stably expressing EGFP-TEV-B56δ (WT) and analyzed the presence of endogenous C in anti-HA immunoprecipitates from the tobacco etch virus–cleaved (TEV-cleaved) eluates of GFP-trapped B56δ (Figure 4B). While C subunit was clearly present in B56δ-Aα (WT) complex (as expected from normal holoenzyme formation), it was barely detectable in the B56δ-Aα-P179L complex, indicating that mutant Aα-P179L may bind B56δ but not C. In contrast, the B56δ-Aα-R182W and B56δ-Aα-R258H complexes still bound a significant amount of C (Figure 4B). However, measurements of specific PP2A activity in these samples showed a decrease in phosphatase activity for all Aα mutants compared with WT (Figure 4C), suggesting that the C subunit present in these B56δ-mutant A-C complexes is catalytically impaired.

Because previous reports had indicated increased B56δ subunit degradation upon gradual reduction of A subunit levels (8, 9), we examined the stability of our mutant subunits, since unstable mutant proteins would preclude the interpretation of our functional data. In line with our hypothesis, we did not experience any problems expressing ID-associated B56δ subunits in cells (Figures 2–4). Furthermore, protein-synthesis–blocking experiments showed that the ID-associated B56δ (P53S, E198K) or Aα (P179L, R182W) mutants tested appeared more long-lived than the WT subunits in our assay (Supplemental Figure 2).

Taken together, for all but one (P53S in *PPP2R5D*) of the de novo mutations identified, our biochemical data are consistent with a common defect in PP2A-B56δ–dependent dephosphorylation. A-C binding–defective B56δ mutants, or C binding– but not B56δ binding–defective Aα-mutants, and B56δ binding Aα-mutants harboring a catalytically impaired C subunit, may all block dephosphorylation of PP2A-B56–specific substrates and interfere with phosphorylation-dephosphorylation dynamics in the brain. In line with this hypothesis, overexpression of the E198K B56δ mutant or the R182W Aα mutant in HEK293 cells resulted in increased phosphorylation of GSK-3β Ser9, an established PP2A-B56δ substrate in this cell line (Figure 5 and ref. 10).

Discussion

The presented work demonstrates that de novo missense mutations in genes encoding PP2A subunits may cause syndromic ID — and

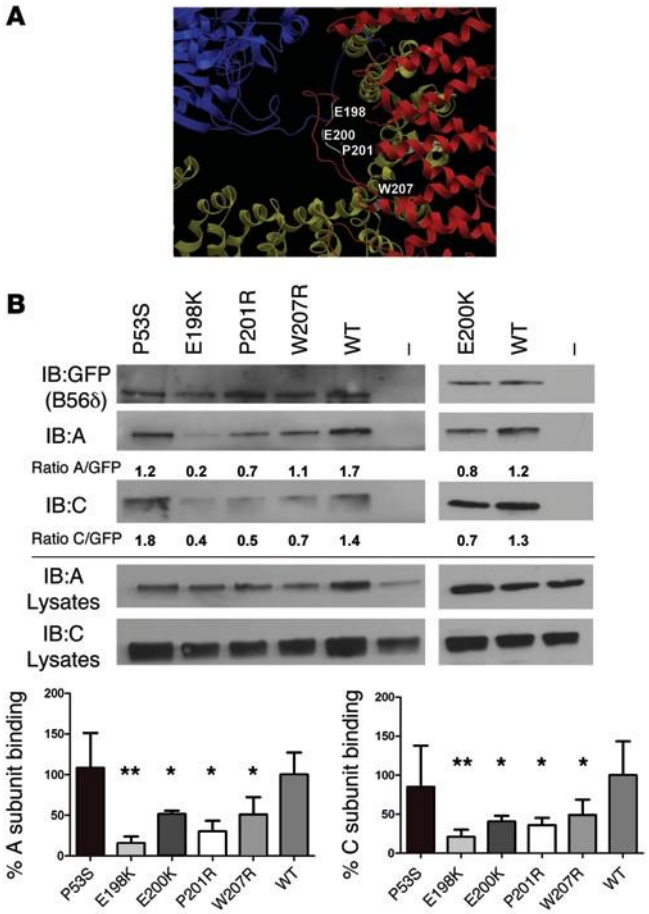

Figure 2. Binding of mutant and WT B56δ to the A or C subunits. (**A**) Model of the highly conserved B56 acidic loop, harboring 4 ID-associated B56δ missense mutations, based on crystallographic data from PP2A-B56γ (PDB code: 2IAE)(5). The corresponding residues in B56γ (highlighted in white) are displayed with amino acid numbering according to B56δ. Note that the E198 residue directly contacts the C subunit. Color code: C subunit, blue; A subunit, yellow; B56γ, red. The structure was analyzed and visualized with Molsoft MolBrowser 3.7. (**B**) Cellular binding assays of ID-associated B56δ mutants and endogenous A and C subunits. EGFP-tagged WT B56δ, 5 ID-associated B56δ mutants (P53S, E198K, E200K, P201R, and W207R), or EGFP alone (–) were ectopically expressed in HEK293 cells. Following EGFP trapping, the presence of endogenous A and C subunits in the trapped complexes was examined by immunoblotting (IB). After quantification of the band intensities with ImageJ software, the ratios between EGFP and C signals — and between EGFP and A signals — were determined and calculated relative to B56δ WT control. Mean values and a representative image of 4 independent experiments are shown (1-way multiple-comparisons ANOVA; $*P < 0.05$, $**P < 0.01$).

probably also nonsyndromic ID, since the facial dysmorphism in these cases is subtle (Figure 1). The *PPP2R5D* and *PPP2R1A* mutations disrupt B56δ-dependent dephosphorylation dynamics and link PP2A dysfunction to congenital brain dysfunction.

In general, the Aα cases were more severely affected than the B56δ cases. All had severe ID, absent speech, diminished brain growth, and partial or complete agenesis of the corpus callosum (Table 3). This is in line with the expected greater difficulty to compensate for a general scaffolding (A) subunit dysfunction than a specific regulatory (B) subunit dysfunction, as reflected by our biochemical data showing additional loss or reduction of holoenzyme assembly of many different PP2A complexes (B55α, B55β, B56α, B56γ, and B56ε) for these Aα mutants (Figure 3B). The Aα scaffolding subunit is highly flexible, composed of 15 tandem repeat HEAT motifs (11) that mediate interactions with a regulatory B subunit (HEAT repeats 1–8) and the C subunit (HEAT repeats 11–15)(4, 5, 12, 13). Two ID-associated *PPP2R1A* mutations (P179L and R182W) cluster in HEAT domain 5 of Aα, and one (R258H) occurs in HEAT domain 7; these mutations are involved in contacts with subunits of all B families (4, 5, 12, 13). However, for these mutations, loss-of-function might be less critical than substrate protection and altered phosphorylation dynamics; hence, the retained binding to PR72 for Aα-P179L could have additional functional consequences due to sequestration of PR72 in a complex deficient in C subunit binding or with decreased specific activity of C.

Besides its scaffolding function, Aα is a major player in the biogenesis of active PP2A holoenzymes (14). This highly regulated but incompletely understood process does not only involve simple trimeric assembly of the A, B, and C subunits, but it also involves several activation steps of the C subunit, which is de novo translated as an inactive enzyme (15). It has been suggested that some of these activation steps require or are facilitated by the A subunit (16, 17), explaining why A-subunit mutations may affect the specific activity of the associated C subunit, as observed here within the B56δ-(mutant A)-C complexes (Figure 4C). Additional activity measurements performed directly in anti-HA immunoprecipitates of HA-tagged (mutated) A subunits seem to further confirm this hypothesis (Supplemental Figure 3). Hence, it can be further rationalized why mutations in the A subunit have a much more severe effect on the PP2A system as a whole, as opposed to mutations in B56δ, which affect a single PP2A holoenzyme complex.

PPP2R5D encodes the longest isoform of the B′ family of PP2A regulatory subunits and harbors unique N- and C-terminal extensions, which are predicted to be important for substrate recognition and/or subcellular targeting (18). Ten out of 11 *PPP2R5D* mutations were located in a conserved acidic loop of B56δ needed for holoenzyme formation (Table 1), and all mutations introduced a positively charged residue (either arginine or lysine). Only one mutation (P53S) was atypical, and this case also had a different clinical picture: it was the only *PPP2R5D* case with short stature and microcephaly (Table 2). In theory, P53S in the B56δ-specific N-terminal domain might change the PP2A-B56δ interaction with relevant substrates or introduce a new phosphorylation site that affects regulation by protein kinases. Such changes could easily have a gain-of-function or dominant-negative effect.

We also observed a correlation between the degree of biochemical disturbance and clinical severity. Among the *PPP2R5D* cases (Table 2), the 6 patients with E198K mutations were the most severely affected, in line with a near absence of A and C subunit binding. The least-affected individuals were the E200K cases, both with mild ID correlating with some residual A and C binding capability (Figure 2B). Notably, E198 is the only one of the 5 mutated residues that directly interacts with the catalytic subunit (Figure 2A and refs. 4–6). Additionally, since all B subunits,

Table 3. Clinical features in cases with de novo *PPP2R1A* missense mutations

	Case 12	Case 13	Case 14	Case 15	Case 16
Mutation	P179L	R182W	R182W	R182W	R258H
Age of examination	3½ yr	4 yr	11 yr	1 yr	5 yr
Sex	Female	Female	Male	Female	Male
Delivery	Normal	Normal	Normal	Labor-induced (hydrocephalus)	Normal
Birth weight	Normal	Normal	Normal	Normal	Normal
Hypotonia	Present	Present	Present	Present	Present
Walked unsupported	Not so far	Not so far	6 yr	Not so far	3 yr
Ataxic gait	N/A	N/A	Yes	N/A	Yes
Language	No words	No words	No words	No words	~30 words
Epilepsy	No	Yes	Yes, multifocal	Yes	Yes
EEG	–	Abnormal	Abnormal	Abnormal	Abnormal
ID	Severe	Severe	Severe	Severe	Severe
Height	~90th	~97th	50th	75th	90th
Head circumference	2 cm < 3rd	10th	2nd	98th > 75th	1 cm < 2nd
Weight	75th	97th	10th	16th	97th
Brain MRI	CC agenesis	CC hypoplasia Large ventricles	CC hypoplasia	CC agenesis Delayed myelinisation Large ventricles	CC hypoplasia Delayed myelinisation
Other findings	Cortical visual impairment	Scoliosis	Scoliosis Hip dysplasia Hypermobility Physically strong Gastrostomy age 8 yr	Cortical visual impairment Unilateral postaxial polydactyly Unilateral kidney agenesis Absent uterus and vagina	Hyperactivity Obstipation Entropion of eyelids

Height, head circumference, and weight are given relative to centiles (the 3rd and 97th centile correspond to ± 2 SD). Abbreviations: y, years; CC, corpus callosum; C/S, cesarean section; –, unknown or not done.

except B56δ and PR72, have been shown to make stabilizing contacts with the C subunit tail (5, 19), C-subunit binding to B56δ, and possibly PR72, may be particularly Aα dependent.

A brain-restricted phenotype is not unexpected for B56δ mutations because *PPP2R5D* is expressed mainly in the brain, particularly in the striatum (18, 20, 21). The brain-restricted phenotype of the Aα mutations (P179L, R182W, and R258H) is more unexpected, since *PPP2R1A* is generally expressed as a common scaffolding subunit for many different PP2A holoenzymes (22). Nevertheless, other malformations than severe corpus callosum hypogenesis were not found in the 5 *PPP2R1A* cases (Table 3). The restricted phenotype may be related to our finding that mutated A subunits bound most tightly to B56δ and (for P179L) also PR72 (Figure 3B), both of which are expressed in the brain, notably in the striatum where both B56δ and PR72 (encoded by *PPP2R3A*) control the dephosphorylation of the neural dopamine-regulated inhibitor of PP1 (DARPP-32) (23–25).

Despite the severe intellectual dysfunction in most patients, B56δ does not appear essential for mammalian brain development, since *Ppp2r5d* knockout mice have intact learning and memory despite ataxia and tauopathy (21). This also suggests that our patients' ID was not caused by haploinsufficiency or a mere loss of function. It is therefore tempting to speculate that the mutated B56δ subunits may not only interfere in a dominant manner with dephosphorylation of B56δ binding PP2A substrates, as shown for GSK-3β in HEK293 cells (Figure 5), but also with subcellular anchoring of PP2A via B56δ, and thereby with control of localized signaling. Thus, A-Cbinding–deficient B56δmutantsmaystillform complexes with B56δ partners, but without promoting dephosphorylation. Such dysphosphorylation may have far-reaching consequences for regulation of localized signaling. One example could be the signaling complex scaffolded by the neural variant of the cAMP-dependent PKA anchoring protein mAKAP, that binds B56δ and several other phosphatases (PP1, PP2B) and kinases (PKA, PDK1, RSK3, ERK5) (26, 27). Dephosphorylation of PP2A-B56 substrates may also be hindered, e.g., the transcription factor and PKA-substrate HAND (28), the neural cyclin-dependent kinase 5 (CDK5) activator CDK5R1 (21), and DARPP-32 (24, 25). PKA activates DARPP-32 directly by Thr-34 phosphorylation and indirectly by PP2A-B56δ–dependent activation through Thr-75 dephosphorylation (24). B56δ has several sites for PKA phosphorylation that activate PP2A-B56δ phosphatase activity (24, 29). Moreover, PKA-activated PP2A-B56δ–dependent dephosphorylation of another DARPP-32 phosphorylation site (Ser-97) induces nuclear import — mediating dopamine-dependent epigenetic functions (25) — and lack of nuclear PP2A-B56δ targeting has been associated with juvenile myoclonic epilepsy (30). There is also evidence that PP2A-B56δ regulates both expression (30) and activity (31) of tyrosine hydroxylase, the rate-limiting enzyme in catecholamine synthesis. Which of these candidate substrates will eventually be of pathologic relevance in our ID patients remains to be further determined in appropriate cellular and animal models. A model for a common dominant-negative effect of mutant B56δ and Aα subunits is depicted in Figure 6. This model explains whyall our biochemical findings are compatible with B56δ-dependent PP2A dysregulation. Our model is also supported by a recent paper showing that Aα can form a tight complex in vitro with members of the B'' subunit family, including PR72, in

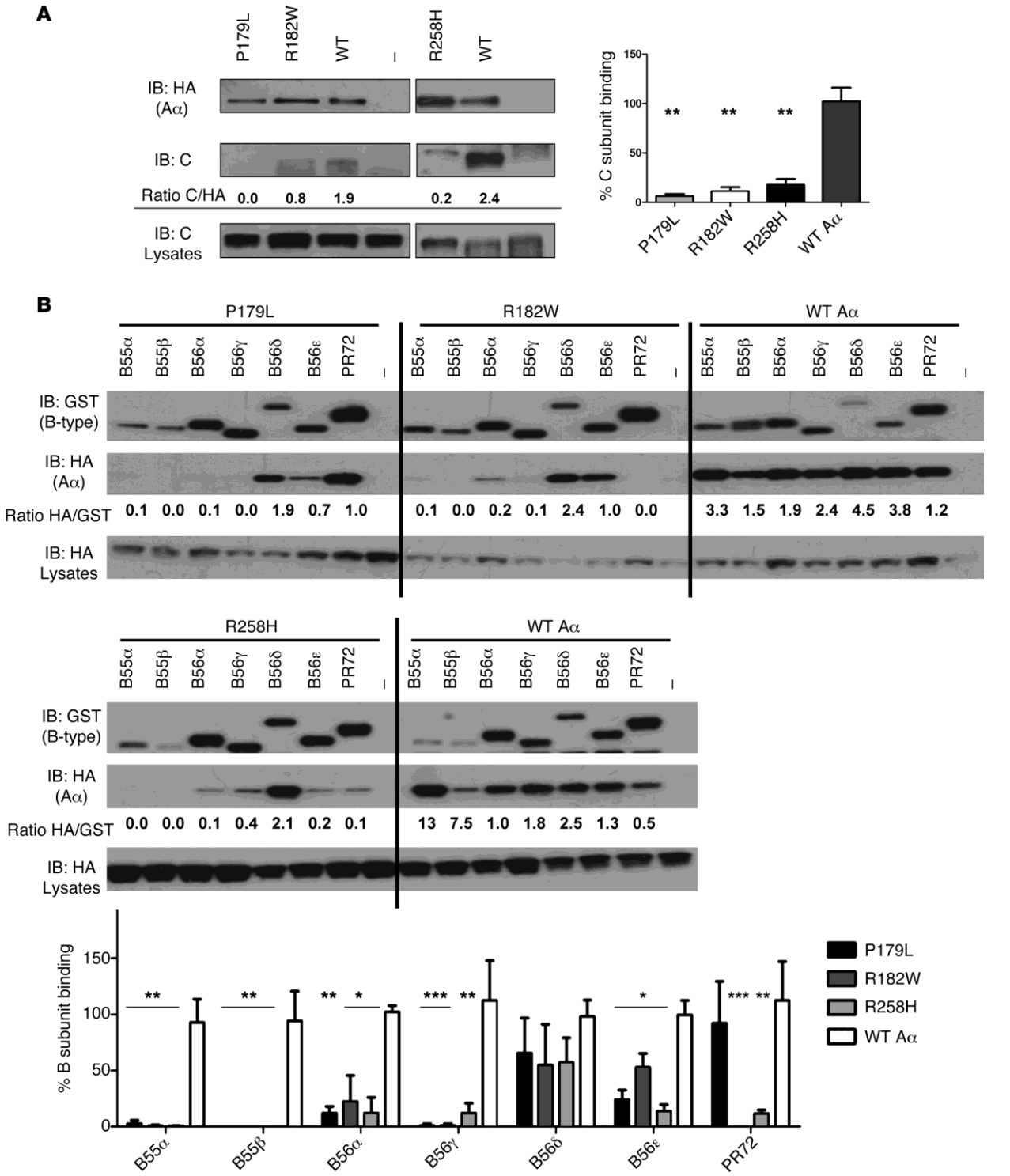

Figure 3. Binding of mutant and WT Aα to C and B subunits. (**A**) PP2A-C subunit binding assays: HA-tagged WT Aα, 3 ID-associated Aα mutants (P179L, R182W, and R258H), or an empty HA-vector (–) were transfected into HEK293 cells. Following anti-HA immunoprecipitation, the presence of endogenous C subunit in the immunoprecipitates was examined by immunoblotting (IB). After quantification of the band intensities with ImageJ software, the ratios between HA and C signals were determined and calculated relative to WT Aα control. Mean values and a representative image of 3 independent experiments are shown (1-way multiple-comparisons ANOVA, $^{**}P < 0.01$). (**B**) PP2A B subunit binding assays: Several GST-tagged B subunits, belonging to 3 different families (B55 or B, B56 or B′, and B′′) or GST alone (–) were coexpressed in HEK293 cells with HA-tagged WT Aα, or ID-associated Aα-P179L, R182W, and R258H mutants. The presence of HA-Aα (WT or mutant) in the complete lysates and the isolated GST pulldown complexes was determined by IB. After quantification of the band intensities with ImageJ software, the ratios between GST and HA signals were determined and calculated relative to WT Aα control (which were set to 100% for each B-type subunit pulldown). Mean values and a representative image of 3 independent experiments are shown (1-way multiple-comparisons ANOVA; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

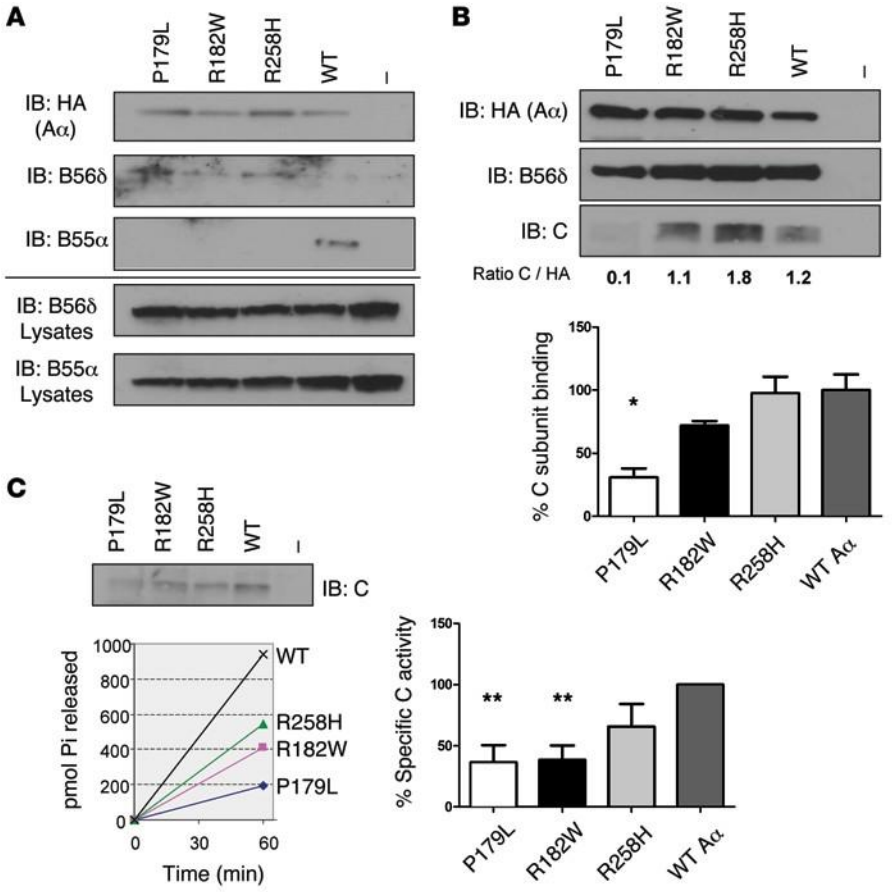

Figure 4. Analysis of Aα mutant complexes. (**A**) Endogenous B subunit binding assays: HA-tagged WT Aα, Aα mutants (P179L, R182W, and R258H), or an empty HA-vector (–) were transfected into HEK293 cells. Following anti-HA immunoprecipitation, presence of endogenous B56δ and B55α subunits in the immunoprecipitates was examined by immunoblotting (IB). (**B**) Formation of B56δ-(mutant Aα)-C complexes: HEK293 cells stably expressing EGFP-TEV-B56δ were transfected with HA-Aα, HA-Aα mutants, or empty HA-vector (–). Following EGFP-trapping and cleavage of the trapped complexes with TEV protease, the eluates were subjected to HA immunoprecipitation and the immunoprecipitates analyzed by IB with anti-HA, anti-C, and anti-B56δ antibodies. After quantification of the band intensities (ImageJ), the ratios between C and HA signals were determined and calculated relative to WT Aα control (set to 100% in each IP-on-IP experiment). Mean values and a representative image of 4 independent experiments are shown (1-way multiple-comparisons ANOVA; $*P < 0.05$). (**C**) PP2A activity measurements in B56δ-(mutant Aα)-C complexes. The pmol number of released phosphate from K-R-pT-I-R-R phosphopeptide (350 μM) was determined by Malachite Green for each B56δ-(mutant Aα)-C complex (retrieved as in **B**). To obtain specific C activities, this number was divided by the amount of C in the respective samples, as determined by IB and following quantification by ImageJ software. All specific activities were eventually recalculated relative to WT Aα control (set to 100%). Mean values and one representative image of 3 independent experiments are shown (1-way multiple-comparisons ANOVA; $**P < 0.01$).

the absence of the C subunit (32). It may be of interest to study if fingolimod (FTY720), a PP2A activator and immunosuppressant that is licensed for treatment of multiple sclerosis (33–35), or FDA-approved compounds of the phenothiazine family that were recently discovered as PP2A activators (36) may improve brain function in these patients.

For future evaluation of de novo mutation origin (paternal or maternal)(37), it is of interest that all 3 *PPP2R1A* mutations also are found in the Sanger Institute's Catalogue of Somatic Mutations in Cancer (the COSMIC database; http://cancer.sanger.ac.uk/cosmic), mainly in endometrial and ovarian cancers (38, 39). Aα P179L/P179R, R182W, and R258H are by far the most prevalent mutations. A growth advantage may also explain mutation recurrence if these de novo mutations turn out to be solely paternal (40). Since 88% of the cancer-associated Aα mutations are of the missense-variant, a dominant-negative effect also in cancer promotion is likely. None of our patients have been diagnosed with or treated for cancer. The cancer risk might not be increased, in line with what is usually the case for congenital gain-of-function mutations in other cancer-related pathways like the RAS/MAPK pathway or the PI3K/AKT cascade. Only further patients and patient follow-ups will answer this question, but a major cancer risk seems unlikely.

The tumor-suppressor effect of PP2A may operate by KRAS/MAPK cascade inhibition, KRAS/ARF/TP53 cascade inhibition, or PI3K/AKT/TP53 cascade inhibition (33). Somatic mosaic activation of the PI3K/AKT cascade causes the megalencephaly-capillary malformation-polymicrogyria (MCAP) and megalencephaly-polymicrogyria-polydactyly-hydrocephalus (MPPH) group of overgrowth syndromes (41). These patients have variable ID, a tendency to develop hydrocephalus and epilepsy, and dysmorphic facial features, including frontal bossing with hypotonia, tented upper lip, and deep-set eyes. The latter features are shared with several of our patients (Figure 1). It is therefore relevant to consider B56δ-dependent PP2A dysregulation syndrome (which we propose to be designated B56deltopathies) among the differential diagnoses to the MCAP/MPPH group of syndromes, at least in some cases. It is conceivable that the B56δ mutations may affect only a subgroup of PP2A substrates located distally in the PIK3 signaling cascade — such as GSK-3β Ser9, a well-established Akt phosphorylation site — since the proximal steps do not appear to be subject to B56δ-dependent dephosphorylation (42).

In summary, we have demonstrated that de novo missense mutations in the *PPP2R5D* and *PPP2R1A* genes encoding PP2A subunits represent a new mechanism for ID, due to disrupted B56δ-dependent dephosphorylation dynamics and PP2A dysfunction.

Methods

Case detection. There were 7 Dutch patients (from RUMC: cases 1, 8–9, and 11; from UMC Utrecht: cases 7, 15, and 16). Six of these cases were identified through routine diagnostic exome sequencing

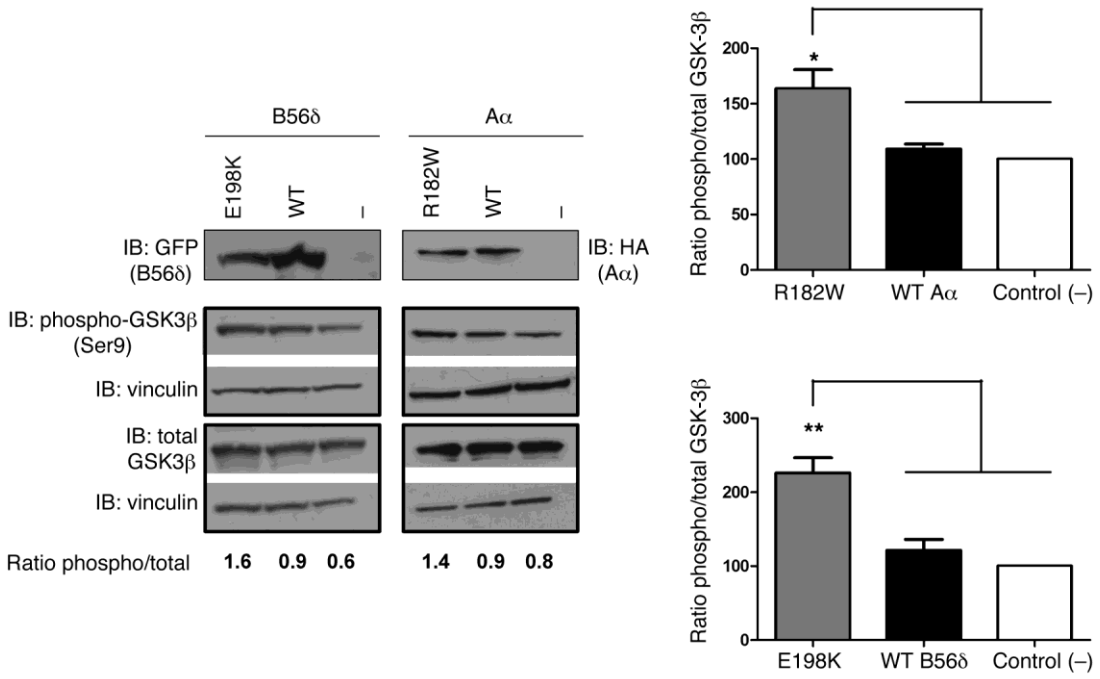

Figure 5. Increased phosphorylation of GSK-3β Ser9 upon expression of B56δ-E198K or Aα-R182W. GFP-tagged WT B56δ, E198K mutant B56δ, or GFP alone (–) (left); or HA-tagged WT Aα, R182W mutant Aα, or HA alone (–) (right) were expressed in HEK293 cells, and the effect on GSK-3β phosphorylation determined by immunoblotting (IB) with the indicated antibodies. Total GSK-3β and phospho-Ser9 GSK-3β signals were determined on different blots, which were both developed for vinculin to correct for loading differences. In cells expressing the ID-associated mutants, higher phospho/total GSK-3β ratios were found, relative to cells transfected with empty expression vector (in which case, this ratio was set to 100%) or cells expressing WT subunits. Mean values and one representative image of 3 independent experiments are shown (1-way multiple-comparisons ANOVA; $*P < 0.05$, $**P < 0.01$).

of patient-parent trios. These patients showed no pathogenic copy number changes (high-resolution copy number array). Putative de novo variants were validated by Sanger sequencing of blood DNA. In 4 of 7 cases, other de novo variants were identified, but none of these were likely to cause the phenotype (Supplemental Table 1). A possible exception was case 16, in which heterozygosity for a likely pathogenic mutation in the *TMEM67* was identified. The patient had clinical features partly consistent with a ciliopathy (unilateral postaxial polydactyly, unilateral kidney agenesis, and absent uterus). However, a second *TMEM67* mutation was not identified after Sanger sequencing. Cases 1 and 11 have been previously published as part of studies showing the power and impact of next-generation sequencing–based (NGS-based) technologies in a clinical diagnostic setting without clinical details on the patients' phenotypes or functional evaluation of the mutations (43, 44). Case 8 was identified as part of large-scale resequencing study of candidate ID genes using molecular inversion probes (MIPs). *PPP2R5D* was one of 42 candidate ID genes tested in 1,300 cases with a clinical diagnosis of ID and in whom previous molecular diagnostic tests were negative.

The 7 United Kingdom patients (cases 3–5, 10, and 12–14 in Tables 2 and 3) were recruited to the DDD study by the United Kingdom National Health Service or the Republic of Ireland Regional Genetics Service (3). Recruitment criteria were patients with neurodevelopmental disorders and/or congenital anomalies, abnormal growth parameters, dysmorphic features, and unusual behavior. DNA samples from patients and parents were analyzed by the Wellcome Trust Sanger Institute using high-resolution microarray analysis (array-CGH and SNP-genotyping) to investigate copy number variations (CNVs) in the child, and exome sequencing to investigate single nucleotide polymorphisms (SNPs) and small insertions/deletions (indels). Putative de novo sequence variants were validated using targeted Sanger sequencing of blood-sample DNA. All genomic variants were annotated with the most severe consequence predicted by Ensembl Variant Effect Predictor (VEP) (45) and their minor allele frequencies observed in diverse population samples. Likely, diagnostic variants were fed back to referring clinical geneticists for validation and discussion with the family via the patient's record in Database of Chromosomal Imbalance and Phenotype in Humans Using Ensembl Resources (DECIPHER; Ensembl) (46), where they can be viewed in an interactive genome browser. Full genomic datasets were also deposited under accession number EGAS00001000775 in the European Genome-Phenome Archive (www.ebi.ac.uk/ega).

The de novo *PPP2R5D* mutations in the Norwegian (case 2) and Israeli (case 6) patients were identified by exome sequencing of parent-child trios in a diagnostic setting. Only the *PPP2R5D* variant remained as true de novo after filtering and verification by Sanger sequencing of blood DNA, and no pathogenic copy number changes were detected by a high-resolution copy number array.

Biochemical investigations. To study the functional consequences of the de novo missense mutations, WT Aα and B56δ (isoform 1) cDNAs were cloned into HA-tag (pMB001) and EGFP-tag (pEGF- P-C1) eukaryotic expression vectors, respectively. The different PP2A B-subunit cDNAs were in a GST-tag eukaryotic expression vector, as described (19). PCR-based site-directed mutagenesis (Stratagene) was performed directly in the pMB001 or pEGFP vectors with proof-

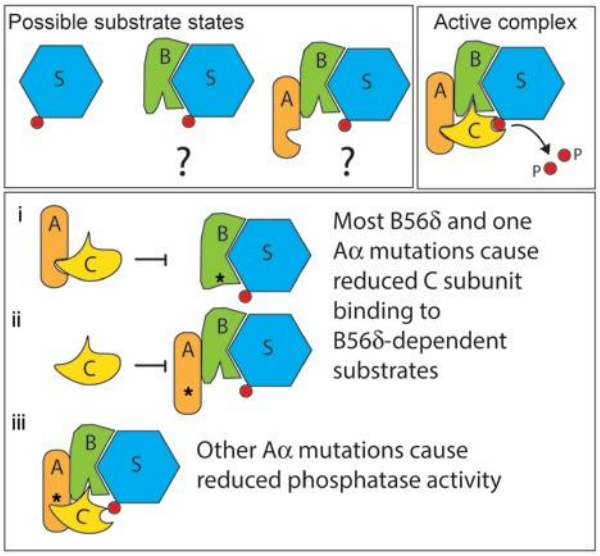

Figure 6. Mechanistic model. Top panels model the physiological situation in which the B-type subunit dictates subcellular targeting, substrate specificity, and substrate dephosphorylation by the C subunit. Conceivably, certain B subunits (like B56δ) or A-B dimers could dock to substrates independent of holoenzymes (alternatives labeled with question marks). Bottom panel displays the pathological situation in which (i) a B subunit mutation hindering interaction of the A and C subunit, (ii) an A subunit mutation hindering interaction of the C subunit but not the B subunit, or (iii) an A subunit mutation resulting in the incorporation of a catalytically impaired C subunit into the trimeric complex result in protection of B subunit–directed substrate dephosphorylation by a competition-based, dominant-negative mechanism. The common feature in all ID cases described here is hindered access of the PP2A activity to B56δ-specific PP2A substrates. S, substrate; P, phosphate.

reading Pwo polymerase (Roche Applied Science) and complementary DNA oligonucleotide primers (Sigma-Aldrich) containing the desired point mutations (primer sequences in Supplemental Table 2). All mutations were confirmed by sequencing (LGC Limited). Thereafter, HEK293 cells (ATCC) were transfected with PEI transfection reagents according to standard protocol. Forty-eight hours after transfection, cells were rinsed with PBS, lysed in 200 μl NET buffer (50 mM Tris pH 7.4, 150 mM NaCl, 15 mM EDTA, and 1% Nonidet P-40) containing protease and phosphatase inhibitor cocktail (Roche Applied Science), and centrifuged for 15 minutes at 13,000 *g*. In case phosphatase activity needed to be measured, phosphatase inhibitors were omitted from the lysis buffer.

For EGFP trapping, cell lysates were incubated at 4°C for 1 hour with wash buffer (10 mM Tris-HCl pH 7.5, 0.5 mM EDTA, and 150 mM NaCl) and 15 μl GFP-trap-A beads (ChromoTek GmbH) on a rotating wheel. The beads were washed 4 times with 0.3 ml of wash buffer.

For GST pulldown, cell lysates were incubated at 4°C for 1 hour with $NENT_{100}$ buffer (20 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.1% Nonidet P-40, 25% glycerol, and 100 mM NaCl) containing 1 mg/ml bovine serum albumin and 25 μl glutathione-Sepharose beads (GE Healthcare) on a rotating wheel. The beads were washed 2 times with 0.3 ml of $NENT_{100}$ containing 1 mg/ml bovine serum albumin, and 2 times with 0.3 ml of $NENT_{300}$ containing 300 mM NaCl.

For HA immunoprecipitation, the lysates were precleared with Protein A-Sepharose beads (GE Healthcare) for 1 hour, and incubated on a rotating wheel at 4°C for 2 hours with 1 μg HA antibody (Sigma-Aldrich) in TBS/1% Nonidet P-40. Protein A-Sepharose beads were added for 1 hour, and beads were washed 2 times in $NENT_{300}$ and 2 times in TBS/0.1% Nonidet P-40. Alternatively, 25 μl HA-agarose beads (Sigma-Aldrich) were directly added to the lysates and incubated on a rotating wheel in 500 μl TBS/0.1% Nonidet P-40 for 1.5 hours at 4°C. Beads were washed 4 times in TBS/0.1% Nonidet P-40.

In all cases, bound proteins were eluted by the addition of NuPAGE sample buffer (Invitrogen) and boiling. The eluted proteins were subsequently analyzed by SDS-PAGE on 4%–12% (wt/vol) Bis-Tris gels (Bio-Rad) and Western blotting. The membranes were blocked in 5% milk solution in TBS/0.1% Tween 20 for 1 hour at room temperature and subsequently incubated with the primary antibody overnight at 4°C. The following primary antibodies were used: mouse monoclonal anti-GST (Sigma-Aldrich), anti-HA (Sigma-Aldrich), anti-GFP (Corning), anti–PP2A-A subunit (supplied by S. Dilworth, Middlesex University, London, United Kingdom), anti–PP2A-C subunit (BD Biosciences); and rabbit polyclonal anti-B55α (Cell Signaling Technology), and anti-B56δ (20). After washing in TBS/0.1% Tween 20, the membranes were incubated at room temperature for 1 hour with horseradish peroxidase–conjugated secondary antibodies (Dako) and developed using a Pierce enhanced chemiluminescence detection system (ThermoFisher Scientific). All densitometric quantifications were done with ImageJ software.

For the IP-on-IP approach, a polyclonal population of HEK293 cells stably expressing EGFP-TEV-B56δ was used (selected with 2 μg/ml puromycin). The EGFP-TEV expression vector was a gift of E. Heroes (KU Leuven, Leuven, Belgium). Forty-eight hours after transfection with HA-Aα (pMB001) or HA-mutant Aα (pMB001), EGFP trapping was performed, and the trapped complexes were incubated overnight at 4°C with 0.2 μg/μl of recombinant TEV protease in TEV cleavage buffer (TBS, 1 mM DTT, 0.5 mM EDTA). Following addition of EDTA (1 mM), PMSF (1 mM), and TLCK (1 mM), the TEV eluates were subjected to HA immunoprecipitation with HA-agarose beads and the washed immunoprecipitates were analyzed by immunoblotting with anti-HA, anti–PP2A-C, and anti-B56δ antibodies (20, 21).

For PP2A activity measurements, the HA-agarose beads were washed once more with 20 mM Tris HCl pH 7.4 plus 1 mM DTT, and finally resuspended in 60 μl enzyme dilution buffer (catalog 20-169, Millipore). All assays were performed with 20 μl of this phosphatase suspension and 4.5 μl of 2 mM stock of K-R-pT-I-R-R phosphopeptide (catalog 12-219, Millipore) for 10–60 minutes at 30°C (still in the linear range of the assay). The released free phosphate was determined by the addition of malachite green solution (10/1 mix of solution A [catalog 20-105, Millipore] and solution B [catalog 20-104, Millipore]). After 15 min incubation at room temperature, absorbance at 630 nm was measured in a multi-channel spectrophotometer. Picomolar amounts of phosphate released were calculated by comparison with a standard curve of known KH_2PO_4 concentrations, as outlined in the manufacturer's instructions (Ser/Thr Phosphatase Assay Kit 1, Millipore). Specific phosphatase activity was obtained by correcting

these absolute values for amount of C present in the samples, as determined by immunoblotting with anti-C antibodies and quantification of the signals by ImageJ software.

For protein-stability analysis, HEK293 cells were transfected with EGFP-B56δ (WT); with EGFP-B56δ-P53S or EGFP-B56δ-E198K mutants (pEGFP-C1); or with HA-Aα (WT), HA-Aα-R182W, or HA-Aα-P179L mutants (pMB001), one 10 cm plate per plasmid. Twenty-four hours after transfection, each 10-cm plate was split over 6 wells on a 6-well plate, in which eventually 50 μM cycloheximide (CHX, Sigma-Aldrich) was added per well to block translation. Following incubation with CHX for different time points (0, 10, and 24 hours), whole-cell lysates were prepared in NET lysis buffer and further analyzed by immunoblotting with anti-vinculin mouse monoclonals (Sigma-Aldrich), anti-HA, or anti-GFP antibodies. Band intensities were quantified using ImageJ software.

Statistics. Statistical analysis of biochemical data was done with 1-way multiple-comparisons ANOVA, and $P < 0.05$ was considered to be significant.

The calculation of the chance likelihood for finding 10 de novo mutations in the same 9–amino acid stretch of B56δ was based on the following assumptions: The target size is $<10^{-6}$ of the total ORF size, the number of random missense changes per generation is on average 1, and the index is removed from the equation. This gives a chance likelihood of less than $(10^{-6})^9$, or $<10^{-54}$, not taking the lack of similar variants in ExAC into account. The chance of finding a similar variant in the whole ID dataset by chance is about 2×10^{-3}, assuming that about 2,000 ID cases were tested. In that case, the phenotype should also be random, and this was not the case.

Study approval. The DDD study has UK Research Ethics Committee (REC) approval (10/H0305/83, granted by the Cambridge South REC, and GEN/284/12 granted by the Republic of Ireland REC). In other cases, ascertainment of patients was part of the clinical routine. All patients' families have consented to publication of clinical findings. Written informed consent was also obtained for publication of all facial photographs presented in Figure 1.

Acknowledgments

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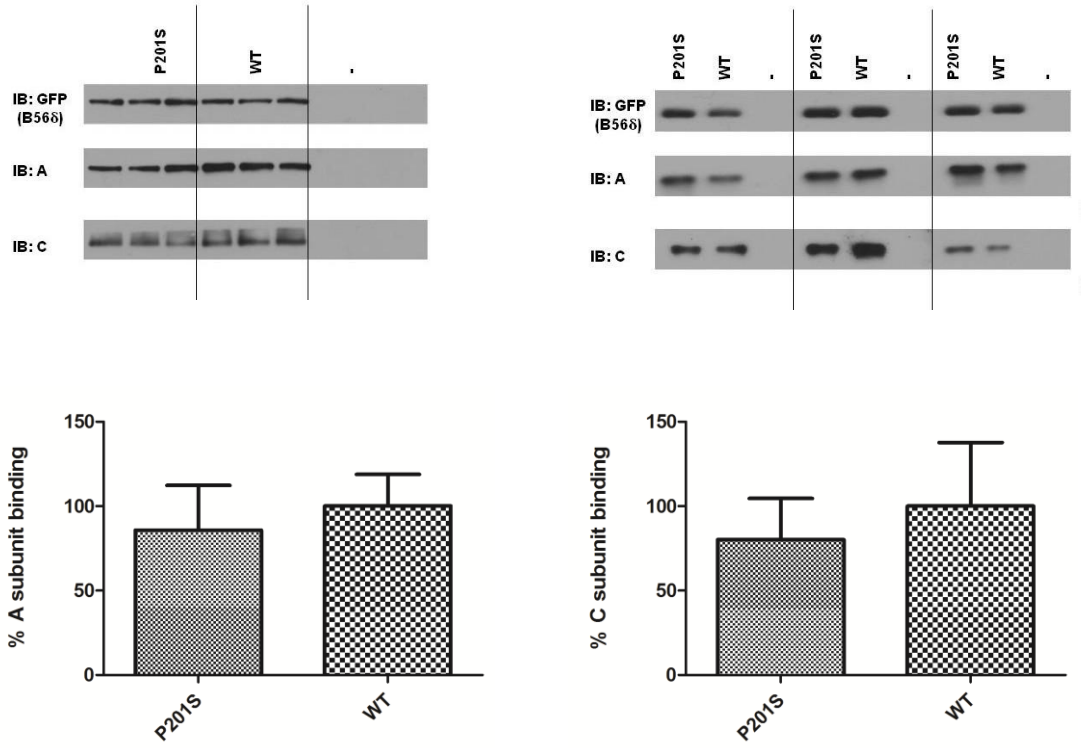
Supplementary Figure 1: Cellular binding assay of the B56δ P201S SNP found in the EVS database.

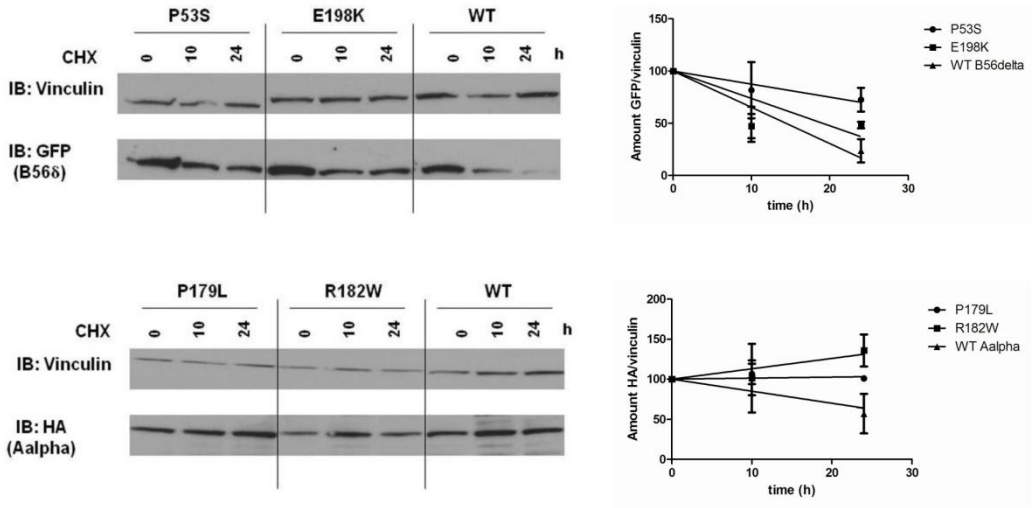
EGFP-tagged wild-type B56δ, B56δ P201S, or EGFP alone (-) were ectopically expressed in HEK293 cells. Following EGFP-trapping, the presence of endogenous PP2A A and C subunits in the trapped complexes was examined by immunoblotting (IB). After quantification of the band intensities with Image J, the ratios between EGFP and C, and between EGFP and A signals were determined, and calculated relative to B56δ wild-type control. The graphs that show A or C binding abilities displays mean values of 6 independent experiments (all shown).

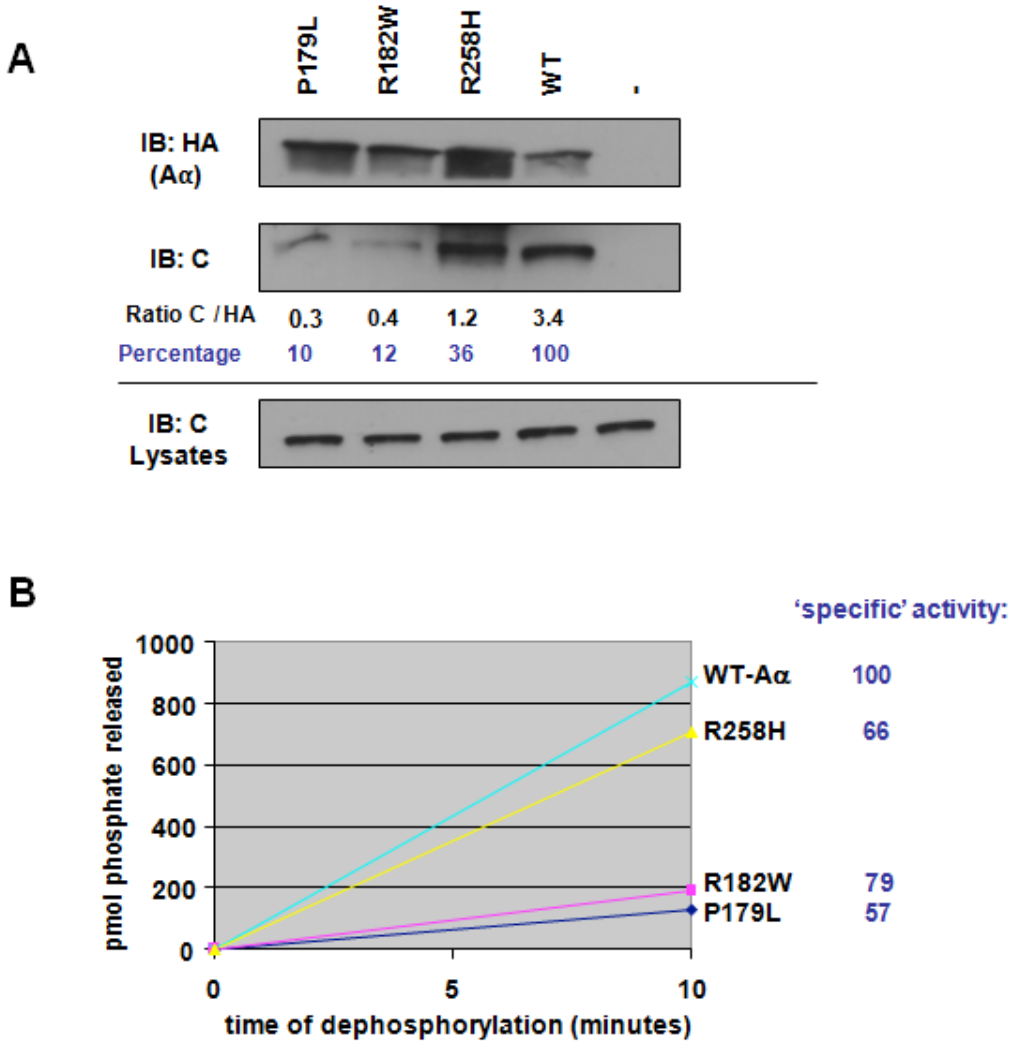


Supplementary Figure 2: Stability of ID-associated B56δ and Aα mutants

HEK293 cells were transfected with EGFP-B56δ (wild-type), EGFP-B56δ-P53S or EGFP-B56δ-E198K mutants (upper panels), or with HA-Aα (wild-type), HA-Aα- R183W or HA-Aα-P179L mutants (lower panels), and incubated with 50 μM cycloheximide (CHX) for different time points (0h, 10h and 24h). After lysis, the protein extracts were analyzed by immunoblotting (IB) with anti-vinculin, anti-HA or anti-GFP antibodies. Band intensities were quantified using ImageJsoftware.

Relative HA/vinculin or GFP-vinculin levels were calculated from three independent experiments and the mean values +/- standard deviation plotted in a graph defined by linear regression (and with relative PP2A subunit/vinculin levels at time point 0h designated as 100%).





Supplementary Figure 3: Reduced specific PP2A C activity in HA-(mutant)Aα-C complexes.

In panel A, binding of C to the HA-tagged (mutant) A subunits is determined (as described in Figure 3A). In panel B, the pmole number of released phosphate from the K-R-pT-I-R-R phosphopeptide (350 μM in assay) was determined by Malachite Green assay for each of the HA-(mutant)A◎-C complexes. The assay was done at 30°C for 10 minutes. Specific C activities were obtained by dividing the absolute amount of pmoles released by the amount of C in the resp. samples, as determined by immunoblotting (IB) and following quantification by Image J. All specific activities were eventually recalculated relative to Aα wild-type control (which was set to 100%).

Supplementary Table 1: Overview of additional exomic alterations ofpotential interest.

Case	Gene	Genomic change	Prediction / evaluation
1	*MEP1B*	*De novo* missense variant	Polymorphism
2	-	No other plausible findings	-
3	*ABCB7D*	Inherited missense variant	Polymorphism
4	*COG1*	Compound heterozygous missense variants	The phenotype was incompatible with CDG type IIG
5	*OCRL*	Inherited missense variant	The phenotype was incompatible with Lowe syndrome
6	*?*	No information available	
7	*TMEM204 SUV420H2*	*De novo* missense variants	No known disease association
8	*?*	Unknown – detected by targeted (MIP) assay	-
9	-	No other plausible findings	-
10	-	No other plausible findings	-
11	*ELMO2*	*De novo* missense variant	No known disease association
12	-	No other plausible findings	-
13	*PKHD1*	Compound heterozygous missense variants	The phenotype was incompatible with ARPKD
14	*LST1*	*De novo* missense variant	No known disease association
15	*TMEM67*	Heterozygous inherited splice mutation	Phenotype was partly reminiscent of a ciliopathy, but a second mutation was not detected.
16	-	No other plausible findings	-

Supplementary Table 2: Oligonucleotides used for site-directed mutagenesis

Primers B56δ mutations	
P53S Forward	5'-GTCTCAGCCATCGTCATCCAAC-3'
P53S Reverse	5'-GTTGGATGACGATGGCTGAGAC-3'
E198K Forward	5'-GACCCAGAGAAAGATGAGCCC-3'
E198K Reverse	5'-GGGCTCATCTTTCTCTGGGTC-3'
E200K Forward	5'-GACCCAGAGGAAGATAAGCCCACCCTGGAAGCTGC-3'
E200K Reverse	5'-GCAGCTTCCAGGGTGGGCTTATCTTCCTCTGGGTC-3'
P201R Forward	5'-GACCCAGAGGAAGATGAGCGCACCCTGGAAGCTGCTTGGCC-3'
P201R Reverse	5'-GGCCAAGCAGCTTCCAGGGTGCGCTCATCTTCCTCTGGGTC-3'
P201S Forward	5'-GACCCAGAGGAAGATGAGAGCACCCTGGAAGCTGCTTGGCC-3'
P201S Reverse	5'-GGCCAAGCAGCTTCCAGGGTGCTCTCATCTTCCTCTGGGTC-3'
W207R Forward	5'-GCCCACCCTGGAAGCTGCTAGGCCACATCTCCAGCTCG-3'
W207R Reverse	5'-CGAGCTGGAGATGTGGCCTAGCAGCTTCCAGGGTGGGC-3'
Primers Aα mutations	
P179L Forward	5'-GCTCAGATGACACCCGCATGGTGCGGCGGGC-3'
P179L Reverse	5'-GCCCGCCGCACCATGCGGGTGTCATCTGAGC-3'
R182W Forward	5'- ACCCCCATGGTGTGGCGGGCCGCA-3'
R182W Reverse	5'- TGCGGCCCGCCACACCATGGGGGT-3'
R258H Forward	5'-AAGACAAGTCCTGGCACGTCCGCTACATGGT-3'
R258H Reverse	5'-ACCATGTAGCGGACGTGCCAGGACTTGTCTT-3'

6

Results (2):

Uterine cancer-associated *PPP2R1A* mutations increase cancer cell growth by a dominant mechanism-of-action

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ABSTRACT

Somatic *PPP2R1A* missense mutation is one of only a few genomic alterations occurring with high frequency in serous endometrial carcinoma and carcinosarcoma, both clinically aggressive subtypes of uterine cancer with few therapeutic options. *PPP2R1A* encodes the scaffolding Aα subunit of protein phosphatase 2A (PP2A), a major family of Ser/Thr phosphatases increasingly recognized to belong to the critical human tumor suppressors. Previously studied cancer-associated Aα mutants exhibited defects in binding to other PP2A subunits and were suggested to contribute to cancer development through a mechanism of functional haploinsufficiency. Here, we report on the biochemical and functional consequences of eleven recurrent endometrial carcinoma-associated *PPP2R1A* mutations clustering into Aα HEAT-repeats 5 and 7. Besides the predicted loss-of-function effects on formation of some PP2A holoenzymes, we uncover two novel mechanisms-of-action, consistent with dominant-negative and gain-of-function effects. Dominant-negative Aα mutants retain binding to specific subunits of the B'/B56 family, but form substrate-trapping complexes with impaired phosphatase activity due to increased recruitment of the PP2A inhibitor TIPRL1. Gain-of-function Aα mutants show significantly increased binding to B'''/STRN and several STRIPAK components. In accordance with these mechanisms, ectopic expression of Aα mutants in endometrial carcinoma cells increases anchorage-independent growth and tumor formation *in vivo*, and causes hyperphosphorylation of oncogenic PP2A-B'/B56 substrates in the Akt and mTOR/p70S6K signaling pathways, while phosphorylation of ERK, a STRIPAK target, was decreased. Our data reveal how *PPP2R1A* mutations affect PP2A function and oncogenic signaling during endometrial cancer development, providing a molecular basis for improved design of targeted therapies.

INTRODUCTION

Although not as common as endometrioid carcinoma (type I), serous uterine carcinoma (type II) is a highly aggressive disease characterized by high mortality due to a tendency for early metastasis and resistance to conventional chemotherapy (1). While genome-wide molecular changes in low grade endometrioid carcinomas have been revealed through The Cancer Genome Atlas research initiative (2), the molecular changes associated with serous endometrial cancer pathogenesis have only recently started to emerge. Several studies have consistently shown that, besides *TP53* mutation (occurring in up to 80-95% of cases), relatively few additional molecular genetic aberrations can be found in this cancer type, the most prevalent being alterations in *PPP2R1A*, *PIK3CA*, *FBXW7, CCNE1* and *CHD4* (3,4,5). Amongst these, *PPP2R1A* appears by far the most frequently altered, in 18.4 up to 43.2% of cases, depending on the study (2,3,4,5,6,7,8,9) (*Suppl. Table S1*). *PPP2R1A* aberrations already occur early during progression in the pre-invasive intraepithelial precursor lesions (3), are distinctive for the serous subtype (6), and clearly distinguish the uterine serous carcinomas from the clinicopathological similar ovarian high-grade serous carcinomas (1,7 ,8). In addition, in the very aggressive uterine carcinosarcoma *PPP2R1A* mutation is a frequent event, occurring in 21.4% up to 26.8% of cases (6, http://www.cbioportal.org/study.do?cancer_study_id=ucs_tcga), as well as in the highly malignant undifferentiated endometrial carcinoma (20% of cases) (10) (*Suppl. Table S1*). *PPP2R1A* encodes the Aα subunit of type 2A protein phosphatases (PP2A), suggesting a prominent, but so far unexplored, role for PP2A in the etiology of these uterine cancers.

PP2A phosphatases are well-recognized to belong to the critical tumor suppressors in a large variety of human cancers (11,12,13). Structurally, these enzymes are highly diverse; they consist of a catalytic C subunit (or PP2Ac), a structural A subunit and one of multiple (at least 23) regulatory B-type subunits, the latter defining substrate specificity and subcellular localization of the holoenzyme (14,15). Since different isoforms exist of A, B and C subunits, their combination can result in at least 96 different PP2A holoenzymes, each with different signaling functions in transcription, translation, DNA replication, responses to extracellular growth and stress factors, apoptosis, and cell cycle control (16,17,18). The PP2A A subunit plays a major role in holoenzyme assembly, as it forms the scaffold between the C and B-type subunits. It is a highly flexible molecule (19), composed of 15 tandemly repeated HEAT (Huntington, Elongation Factor 3, PR65/A, TOR) motifs (20). Biochemical and structural studies have demonstrated that interaction with a regulatory B-type subunit is mediated by HEAT-repeats 1-8, while interaction with the C subunit occurs through HEAT-repeats 11-15 (21,22,23,24,25,26,27,28). Despite these different interaction domains, C and B subunit binding to the A subunit do not necessarily occur independently from each other (29). For instance, N-terminal deletions of Aα inhibit C binding (21), and PR55/Bα and PR61/B'γ3 do not bind to a C-terminally truncated Aα (22,30), while B"/PR72 does (30). This is all indicative for cooperativity between specific B-type subunits and the C subunit in binding the A subunit, while other B-type subunits may bind the A subunit independently from C (31). In particular, it has been shown that the highly conserved C-terminal tail of the C subunit provides additional, stabilizing contacts with B/PR55 and most B'/PR61 subunits, but not B'/PR61δ, B"/PR72 or B"'/striatin subunits, to promote holoenzyme assembly

(25,32,33,34,35). Thus, the determinants governing PP2A holoenzyme assembly seem to be significantly dependent on the nature of the B-type subunit that needs to be incorporated.

While some PP2A complexes may be proto-oncogenic (36,37,38), an overwhelming amount of evidence rather underscores their ability to suppress various (proto)oncogenes and oncogenic signaling pathways (17,39,40,41,42). In accordance, it was found that inhibition of PP2A – together with expression of oncogenic RasV12 – is an absolute requirement to achieve full transformation of a large panel of human epithelial cells immortalized by overexpression of the telomerase catalytic subunit and the SV40 large T antigen (39,42,43,44,45,46), highlighting the major role specific PP2A complexes play in protecting human cells from oncogenic transformation. Hence, there is currently plenty of evidence for deregulation of PP2A activity or expression in a variety of human solid cancers and hematological malignancies (47,48,49,50). Particularly, cancer-associated missense point mutations have been reported in both *PPP2R1A* and *PPP2R1B*, encoding the non-redundant PP2A Aα and Aβ subunits respectively. Inactivating Aβ mutations occur with relatively high frequency in lung (6-15%) (51) and colon cancers (10-15%) (51,52,53), while Aα mutations have less frequently been reported in lung carcinoma (4%), melanoma (7%) and breast carcinoma (2%) (54). Biochemical analyses have shown that most Aβ and all Aα point mutations affect PP2A holoenzyme formation, either by interrupting interaction between A and C subunits, between A and all or specific B-type subunits, or both (39,45,55,56). For the Aα missense mutants characterized so far (E64D, E64G and R418W), these binding defects result in a state of functional haploinsufficiency that promotes cell transformation via activation of the PI3K/Akt pathway (45). In accordance, heterozygous Aα E64D/G knockin mice show increased susceptibility to benzopyrene-induced lung cancerogenesis (57).

Here, we have characterized 11 of the more recently discovered, but not yet biochemically or functionally characterized, *PPP2R1A* missense mutants, identified with high frequency in serous uterine carcinoma and carcinosarcoma (P179R, R182W, R183G/Q/W, R249H, S256F/Y, W257C/G, R258H). All these Aα mutations are heterozygous, cluster in HEAT-repeats 5 or 7, and many of them are recurrent in uterine cancer (*Suppl. Table S1*), but sporadically also occur in other cancer types (58,59). Such high degree of recurrence rather suggests a dominant mechanism-of-action for these mutants, rather than haploinsufficiency. We found that a subset of PP2A holoenzymes is affected by HEAT domain Aα mutants in a dominant-negative manner, whereas another subpopulation of PP2A complexes is affected by a gain-of-function mechanism. Accordingly, expression of HEAT domain Aα mutants in an endometrial cancer cell line harboring two wild-type *PPP2R1A* alleles, triggers activation of p70 S6 kinase and Akt signaling pathways and promotes anchorage-independent growth and tumor growth *in vivo*.

MATERIALS AND METHODS

Site-directed mutagenesis. Wildtype Aα coding region was cloned into an HA-tag eukaryotic expression vector (pMB001). PCR-based site-directed mutagenesis (Stratagene) to generate eleven recurrent serous uterine carcinoma-associated Aα mutants (P179R, R182W, R183G, R183Q, R183W, R249H, S256F, S256Y, W257C, W257G, R258H) and the melanoma-associated R418W mutant was performed directly in the pMB001 vector with proofreading

Pwo polymerase (Roche Applied Science) and complementary DNA oligonucleotide primers (Sigma Genosys) containing the desired point mutations (primer sequences in *Suppl. Table S2*). The Aα deletion mutant containing HEAT repeats 1-10 (Aα aa1-410) was generated by introduction of a stop codon at amino acid 411 (*Suppl. Table S2*). The mutant (P179R, R182W, R183G, R183Q and S256F) and WT Aα coding regions were subcloned in the pLA CMV N-Flag vector via the In-Fusion® HD cloning kit (Clontech Laboratories). All Aα mutations were confirmed by sequencing (LGC genomics). The different PP2A B-type subunit coding regions were cloned in a GST-tag eukaryotic expression vector as described (33). The B'ε coding region was cloned in pEGFP-C1 vector. Bα, B'γ1 and B'δ (60) were additionally subcloned in pEGFP-TEV. *STRN3* cDNA (clone HsCD00623024) was obtained from DNASU repository (The Biodesign Institute/Arizona State University) (61) and subcloned into pEGFP-TEV vector using In-Fusion® HD technology (Clontech Laboratories). *TIPRL* cDNA (isoform 1) was obtained by RT-PCR on mRNA isolated from HEC-1-A cells, and cloned into pGMEX-T, pEGFP-TEV and pET15b vectors.

Lentiviral transduction. HEK293T cells (ATCC, Manassas, VA, USA) were transfected in a 6-well plate with Aα WT or mutant cloned into the lentiviral pLA CMV N-Flag vector, in the presence of pCMV-deltaR8.91 and pMD.G-VSVG, using Turbofect transfection reagent (Thermo Scientific). 24h later, the complete supernatant was used to transduce HEC-1-A endometrial carcinoma cells (ATCC, Manassas, VA, USA) and generate cells stably expressing FLAG-tagged WT Aα or mutant proteins.

Cellular PP2A subunit binding assays. HEK293 cells (ATCC, Manassas, VA, USA) were transfected with appropriate PP2A subunit expression vectors using PEI transfection reagens according to standard protocol. 48h after transfection, cells were rinsed with phosphate-buffered saline (PBS), lysed in 200µl NET buffer (50 mM Tris.HCl pH 7.4, 150 mM NaCl, 15 mM EDTA and 1% Nonidet P-40) containing protease and phosphatase inhibitor cocktail (Roche Applied Science) and centrifuged for 15' at 13,000 g.

For GST pull down (from transiently transfected HEK293 cells) and FLAG pull down (from virally transduced HEC-1-A cells), cell lysates were incubated at 4°C for 1h with $NENT_{100}$ buffer (20 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.1% Nonidet P-40, 25% glycerol, 100 mM NaCl) containing 1 mg/ml bovine serum albumin (BSA) and 25 µl glutathione-Sepharose beads (GE Healthcare) or anti-FLAG M2 affinity gel (Sigma Aldrich Corp.) on a rotating wheel. The beads were washed 2 times with 0.3 ml of $NENT_{100}$ containing 1 mg/ml BSA, and 2 times with 0.3 ml of $NENT_{300}$ containing 300 mM NaCl.

For HA pull down, 25 µl anti-HA agarose beads (Sigma Aldrich Corp.) were directly added to the lysates and incubated on a rotating wheel in 500 µl TBS for 1 hour at 4°C. Beads were washed 4 times in TBS.

For GFP trapping, cell lysates were incubated at 4°C for 1h with wash buffer (10 mM Tris-HCl pH 7.5, 0.5 mM EDTA and 150 mM NaCl) and 15 µl GFP-trap-A beads (Chromotek) on a rotating wheel. The beads were washed 4 times with 0.5 ml of wash buffer.

In most cases, bound proteins were eluted by addition of 2x NuPage sample buffer (Invitrogen) and boiling. The eluted proteins were subsequently analyzed by SDS-PAGE on 4–12% (w/v) gels (BioRad) and Western blotting. The membranes were blocked in 5% milk solution in TBS/0.1% Tween-20 for at least 1h at room temperature. Antibodies used for immunoblotting in this study are: mouse monoclonal anti-GST, anti-HA, anti-FLAG, anti-GFP

and anti-vinculin antibodies (all from Sigma-Aldrich Corp.); mouse monoclonal anti-PP2A-A and anti-PP2A-C (generously supplied by Dr. S. Dilworth, University of Middlesex, London, UK); rabbit polyclonal anti-B'δ antibody (62); rabbit polyclonal anti-B'$\gamma_{2,3}$ and anti-B'ε antibodies (generously shared by Dr. B Hemmings, Friedrich Miescher Insitute, Basel, CH); rabbit polyclonal anti-B'α (Abcam); rabbit polyclonal anti-B subunit (#4953, Cell Signaling Technology); mouse monoclonal anti-STRN3 (Acris Antibodies); and rabbit polyclonal anti-TIPRL1 (Abcam). The following antibodies were purchased from Cell Signaling Technology: rabbit polyclonal anti-phospho-p70S6 kinase (T389), rabbit monoclonal anti-phospho-S6 Ribosomal Protein (Ser235/236), rabbit monoclonal anti-S6, rabbit monoclonal anti-phospho-Akt (T308), rabbit monoclonal anti-Akt, rabbit monoclonal anti-phospho-GSK-3β (Ser9), rabbit monoclonal anti-GSK-3β, anti-phospho-ERK1/2 (Thr202/Tyr204), rabbit polyclonal anti-ERK1/2 and rabbit polyclonal anti-phospho-MEK1/2 (Ser217/221). Immunoblot signals were visualized through horseradish peroxidase-coupled secondary antibodies (DAKO) and enhanced chemiluminescence detection (Westernbright ECL-HRP substrate kit, Isogen Life Science). All densitometric quantifications were done with ImageJ software. For statistical analysis, we applied one-way multiple comparisons ANOVA to the average values of all quantified bands of a given condition on a given gel. P-values below 0.05 were considered to be significant.

"IP-on-IP" approach. HEK293 cells were transiently co-transfected (PEI) with expression vectors for EGFP-TEV-B56δ, and HA-Aα (WT or mutant). 48h after transfection, GFP-trapping was performed, and the trapped complexes were incubated overnight at 4°C with 0.2µg/µl of recombinant Tobacco Etch Virus (TEV) protease in TEV cleavage buffer (TBS, 1mM DTT, 0.5 mM EDTA). Following addition of EDTA (1mM), PMSF (1mM) and TLCK (1mM), the TEV eluates were subjected to HA-pull down with HA-agarose beads, and the washed immunoprecipitates analyzed by immunoblotting and/or assayed for PP2A activity.

PP2A activity measurements. HA-agarose beads from HA pull downs according to the IP-on-IP approach, were washed once more with 20mM Tris.HCl pH 7.4 + 1mM DTT, and finally resuspended in 60µl enzyme dilution buffer (catalog # 20-169, Millipore). All assays were performed with 20µl of this 'phosphatase suspension' and 9 µl of 2mM stock of R-R-A-pT-V-A phosphopeptide for 10 to 60 minutes at 30°C (still in the linear range of the assay). The released free phosphate was determined by addition of malachite green solution (100/1 mix of solution A (catalog # 20-105, Millipore) and solution B (catalog # 20-104, Millipore)). After 15 minutes incubation at RT, absorbance at 630 nm was measured in a multi-channel spectrophotometer. Pmole amounts of phosphate released were calculated by comparison with a standard curve of known KH_2PO_4 concentrations, as outlined in the manufacturer's instructions (Ser/Thr Phosphatase Assay Kit 1, Millipore). Specific phosphatase activity was obtained by correcting these absolute values for amount of C present in the samples - as determined by immunoblotting with anti-C antibodies and quantification of the signals by ImageJ.

Expression and purification of recombinant TIPRL1. N-terminally His-tagged TIPRL1 (pET15b) was expressed in E. coli BL21 cells for 3h at 37°C, and purified from the soluble fraction of the bacterial lysate via metal affinity purification using Ni-NTA beads (Affiland), following the manufacturer's standard protocol. After five washes in 50 mM

Tris.HCl pH 8.0, 300 mM NaCl and 13 mM imidazole, and elution in the same buffer with 250 mM imidazole, the purified protein was dialysed against 20 mM Tris.HCl pH 7.4/ PEG 10,000, and stored in 60% glycerol at -80°C. Final protein concentration was determined by absorbance at 280 nm (NanoDrop 2000, Thermo Scientific).

***PPP2R1A* exome sequencing in HEC-1-A cells.** RNA was isolated using TRIzol reagent (LifeTechnologies), reverse-transcribed with MuLV RT (New England BioLabs Inc.), and PCR-amplified with primers Forw 5'-ATGGCGGCGGCCGACGGCGACG-3' and Rev 5'-TCAGGCGAGAGACAGAACAGTCAG-3' using PWO proofreading DNA polymerase (Roche). The purified fragment (Illustra GFX PCR DNA purification kit, GE Healthcare) was subjected to Sanger sequencing using the same primers (LGC Genomics).

Mass spectrometry experiments. FLAG-tagged WT or mutant Aα were isolated from HEC-1-A transduced cells by FLAG pull down (see above). HEC-1-A cells transduced with an empty pLA vector were used as a negative control. After several wash steps as described above, the FLAG-tagged proteins were eluted by addition of 1.5 µg/µl FLAG peptide (23 aa: MDYKDHDGDYKDHDIDYKDDDDK) in 50mM Tris.HCl pH 8.0, containing 50mM NaCl. The eluates were subjected to trypsin digestion and desalting by C18 ZipTip pipette tips (Millipore) before analysis on a hybrid quadrupole-orbitrap mass spectrometer (Q Exactive, Thermo Fisher Scientific). Relative quantification of the interactomes of mutant versus WT Aα was executed with Progenesis software (Nonlinear Dynamics) incorporating protein identifications obtained with the MASCOT (Matrix Science) search engine using the uniprot_swprot (release 07/15/2015, *Homo sapiens*, 20,279 entries). Proteins with an abundance that was at least 3 times higher in any of the experimental conditions than in the 'FLAG-only' control were considered as true interaction partners (100 proteins) and were retained for further calculations. Only abundances of peptides with a MASCOT score of 25 and above (significance threshold) were taken into account. Resulting protein abundances of interacting proteins in each experimental condition were subsequently normalized according to Aα input abundance. From those values, relative quantification to WT Aα was determined. A HEAT-map of differential interactions was made with GENE-E software.

Soft agar colony assay. $1x10^4$ cells/well were seeded in triplicate into 0.35% top agar/0.5% base agar (Sigma Aldrich) supplemented with 2µg/µl Fungizone (Invitrogen) in 6-well plates. Every 2-3 days, fresh medium (DMEM + 10% fetal calf serum) was dropped on top. After four weeks, colonies were counted (Motic AE31 microscope, 4x magnification).

Xenograft assay. $5x10^6$ cells in 200µl PBS were subcutaneously injected in 6-week-old female NMRI-nu (nu/nu) nude mice. Xylazine (Kompun)/ ketamine (Imalgene) was used as a local anaesthetic. Per condition, three mice were injected in both flanks (n=6). All procedures were approved by the local KU Leuven Animal Ethical Committee (project number P104/2012). The number of tumors was determined approximately one month after injection. Tumor size was calculated using the following formula: tumor $width^2$ x (tumor length/2).

RESULTS

Cancer-associated Aα mutations dramatically affect PP2A holoenzyme formation

All recurrent serous uterine cancer-associated Aα mutations are heterozygous and missense, and cluster into HEAT-repeat 5 (P179L, P179R, R182W, R183G, R183Q, R183W) and HEAT-repeat 7 (R249H, S256F, S256Y, W257C, W257G, R258H). To investigate how these Aα mutations affect PP2A holoenzyme assembly, we first evaluated their binding to PP2Ac. HA-tagged wildtype (WT) Aα, the PP2Ac-binding deficient R418W Aα mutant (45,55) and the 11 uterine cancer-associated Aα mutants were expressed in HEK293 cells, and interaction with endogenous PP2Ac was studied after HA pull down. All mutants, but R249H, show reduced PP2Ac binding, when compared to WT Aα (*Figure 1*).

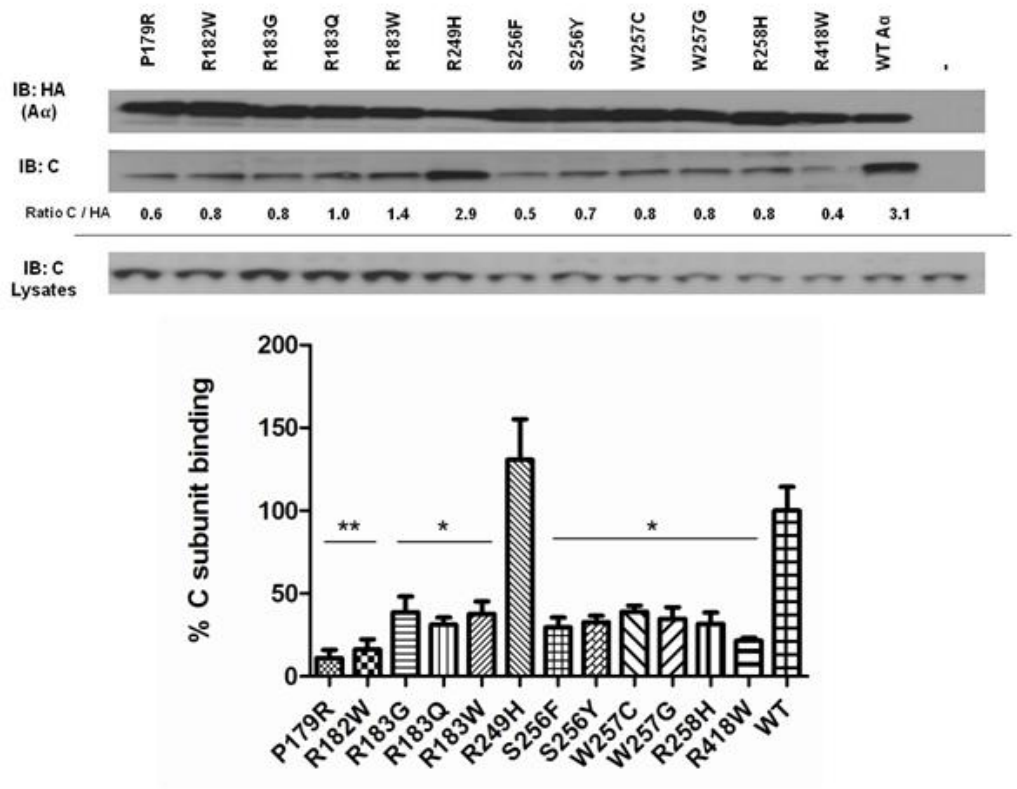


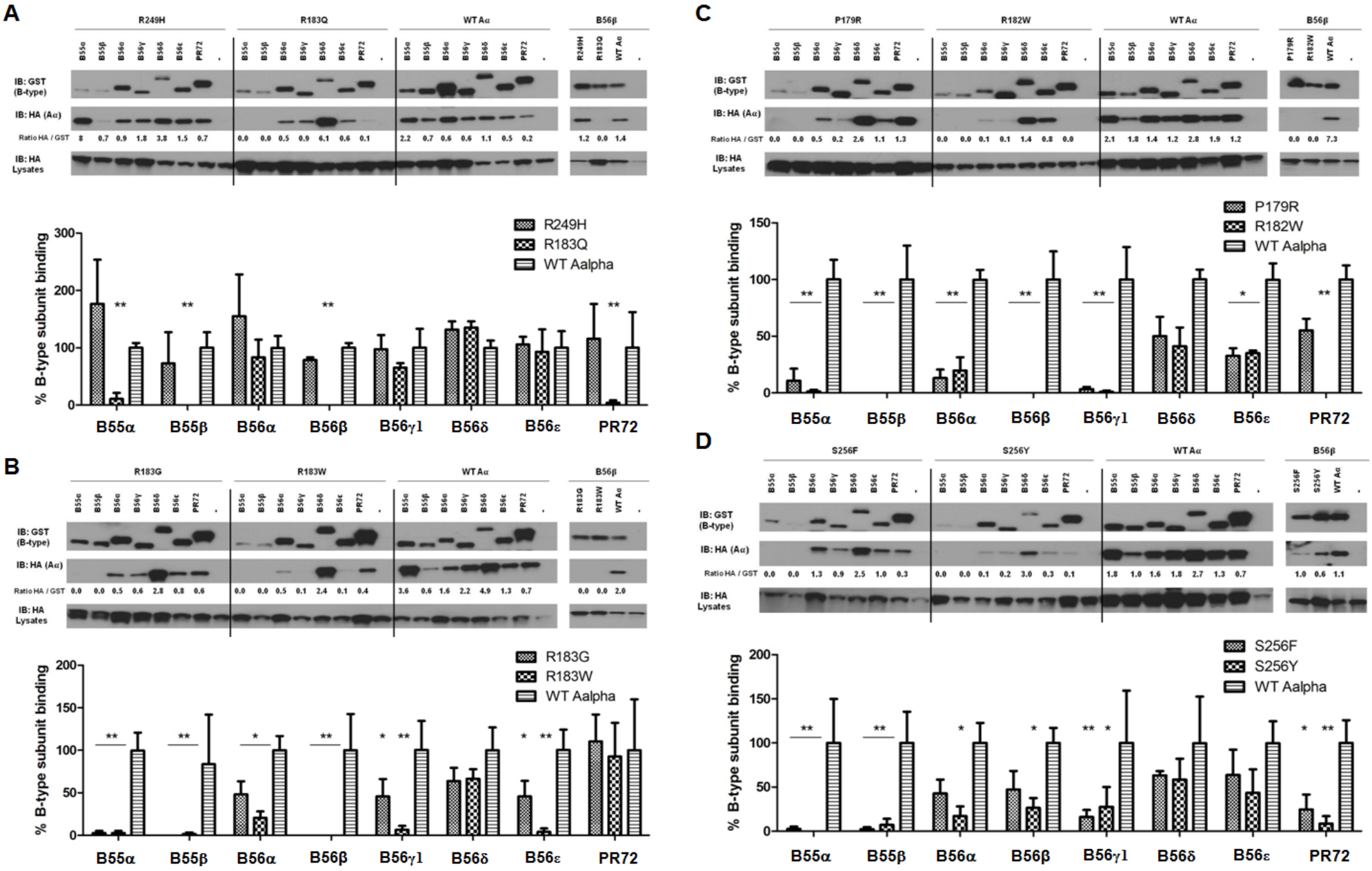
Figure 1: PP2Ac subunit binding to cancer-associated Aα mutants.
WT Aα, the melanoma-associated R418W Aα mutant and 11 endometrial cancer-associated Aα mutants (all HA-tagged), or an empty HA vector (-) were transfected into HEK293 cells. Following HA pull down, interaction with endogenous PP2Ac subunit was examined by immunoblotting (IB). After quantification of the band intensities with Image J, the ratios between HA and C signals were determined and calculated relative to Aα WT control (set to 100%). Mean values and a representative image of four independent experiments are shown (*, $p < 0.05$; **, $p < 0.01$).

Next, we determined binding of the mutant HA-tagged Aα proteins with GST-tagges B-type subunits of the B, B' and B" families in GST pull down assays (*Figure 2*). The single Aα

mutant that retained PP2Ac binding, R249H, also showed normal binding to all B-type subunits tested (*Figure 2A*). All other Aα mutants showed varying binding alterations to at least one B-type subunit (*Figure 2A-2F, Table 1*). The most pronounced defects in binding were observed for the PR55/B family members, Bα and Bβ. In fact, all of the remaining 10 Aα mutants no longer (or hardly, ≤11%) bound to these B subunits anymore (*Table 1*). Also the B′ and B″ family members primarily showed binding defects, but in a much more heterogeneous way as compared to the B family members. For example, PR72/B″ was found to bind efficiently to R183G, R183W and W257C, but it lost binding or bound significantly less (<30%) to R182W, R183Q, S256F, S256Y, W257G and R258H. Binding to P179R was, although decreased by half, not significantly different from the WT (*Table 1*). These data already indicate that a single mutation in either of the two affected Aα HEAT-repeats can have an impact on PR72 binding, and that not just the mutated residue determines the binding deficiency, but also the amino acid in which it is substituted (*e.g.* compare R183G with R183Q).

Similar conclusions could be drawn from the results of the binding experiments obtained for the B′ subunits (*Table 1*), although in these cases, remarkable isoform-specific differences in binding behavior to a single cancer-derived Aα mutant were additionally found. The latter finding is especially clear for the B′δ isoform which overall shows the least binding defects, while B′γ1, showed the most pronounced binding defects in this assay. In fact, none of the binding differences observed between WT and the 10 Aα mutants reached statistical significance for B′δ (although binding to P179R, R182W, R183G, R183W, S256F/Y and R258H was only retained for 40-65%), while B′γ1 lost binding or bound significantly less (<30%) to 8 Aα mutants and to R183G (46%), and only retained binding to R183Q (75%). B′β lost binding (0%) to all HR5 mutants (P179R, R182W and R183G/W/Q) and R258H, bound significantly less (10-30%) to S256Y and W257C/G, and only retained binding to S256F (47%). B′α bound significantly less (≤20%) to 5 Aα mutants (P179R, R182W, R183W, S256Y and R258H), showed moderate binding defects (30-50%) to 3 others (R183G, W257C and W257G), and retained binding to R183Q (85%) and S256F (45%). B′ε showed severe loss of binding to only 2 Aα mutants (R183W and R258H), moderately lost binding (30-50%) to 4 others (P179R, R182W, R183G and W257G), and retained binding to S256F/Y (45-60%), R183Q and W257C (both 100%). The binding defects of the melanoma-associated R418W mutant were largely as reported before (*Figure 2G*) (45,55,63).

Binding of HA-tagged mutant Aα binding to the B‴ subunits was monitored in GFP trapping assays with GFP-tagged STRN3 (SG2NA) (*Figure 3*). Intriguingly, we found that upon STNR3 co-expression, several Aα mutants were significantly more expressed (*Figure 3A,B*), while this was not observed when the mutants were co-expressed with any of the B, B′ or B″ subunits tested (*Figure 2*). This suggested that STRN3 might have a stabilizing effect on the mutants, due to a better binding to this B subunit. Indeed, when we analyzed the GFP-trapped complexes for HA-Aα binding, all but one (R249H) of the Aα mutants tested bound obviously better to GFP-STRN3 as compared with WT Aα (*Figure 3A*). When the increase in binding was normalized to the levels of expression , two Aα mutants, S256F and R258H, were found to statistically significantly bind better to STRN3 as compared with WT Aα (*Figure 3C*).


A
R249H
R183Q
WT Aα
B56β
IB: GST (B-type)
IB: HA (Aα)
Ratio HA / GST
IB: HA Lysates
% B-type subunit binding
R249H
R183Q
WT Aalpha
B55α
B55β
B56α
B56β
B56γ1
B56δ
B56ε
PR72
B
R183G
R183W
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S256F
S256Y
S256F
S256Y

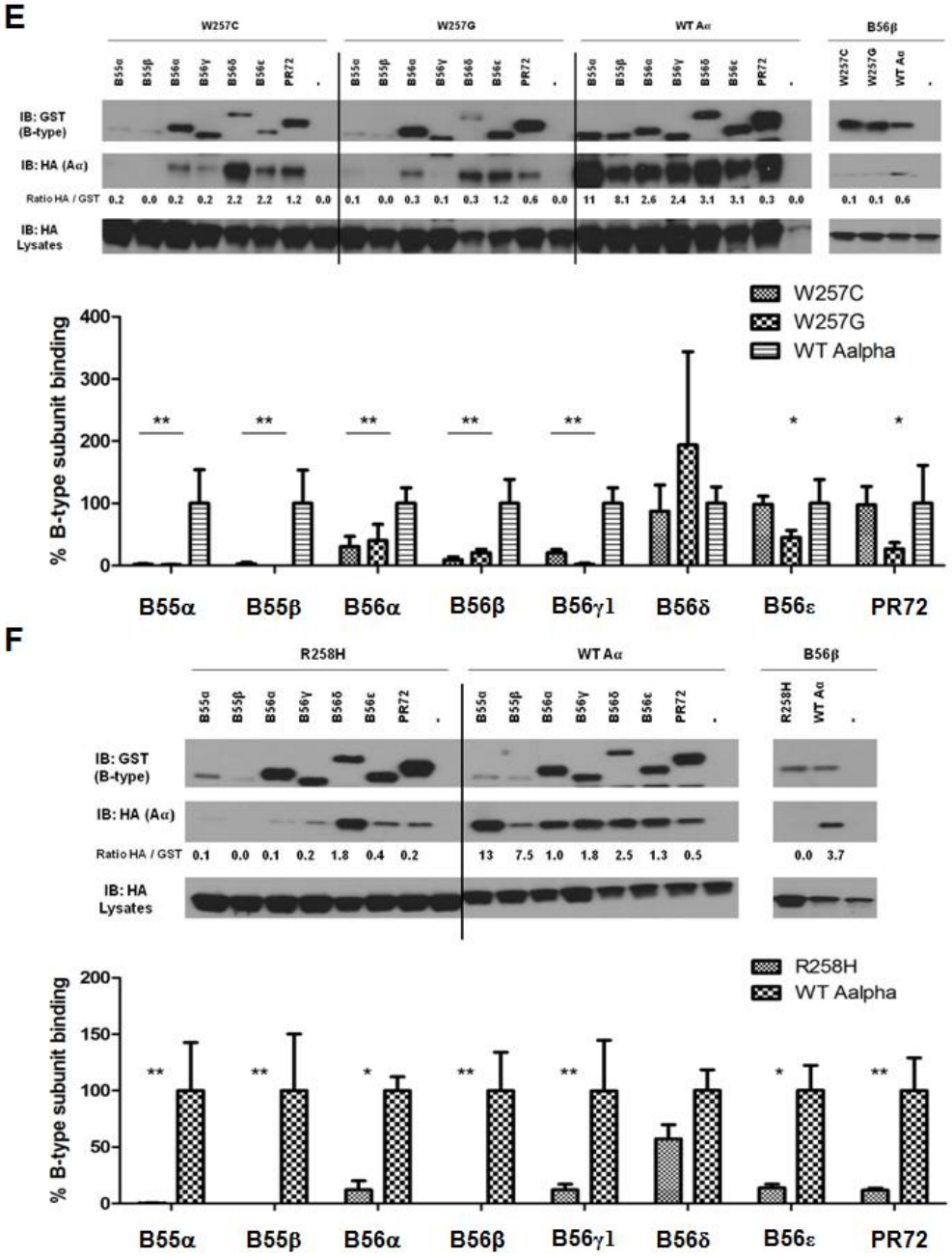


Figure 2: Binding of B-type subunits (B, B' and B") to endometrial cancer-associated Aα mutants.

GST-tagged B subunits or empty GST vector (-) were co-expressed in HEK293 cells with HA-tagged WT or mutant Aα. The following 11 endometrial cancer-associated Aα mutants were tested: R249H and R183Q (panel **A**), R138G and R183W (panel **B**), P179R and R182W (panel **C**), S256F and S256Y (panel **D**), W257C and W257G (panel **E**), R258H (panel **F**) and the previously tested melanoma-associated R418W mutant (45,55) (panel **G**). The HA signal in the complete lysates and in the isolated GST pull down complexes was determined by immunoblotting (IB). After quantification of the band intensities with Image J, ratios were determined between the GST and HA signals and calculated relative to Aα WT control (set to 100% for each B-type subunit pull down). Mean values and a representative image of three independent experiments are shown (*, $p < 0.05$; **, $p < 0.01$).

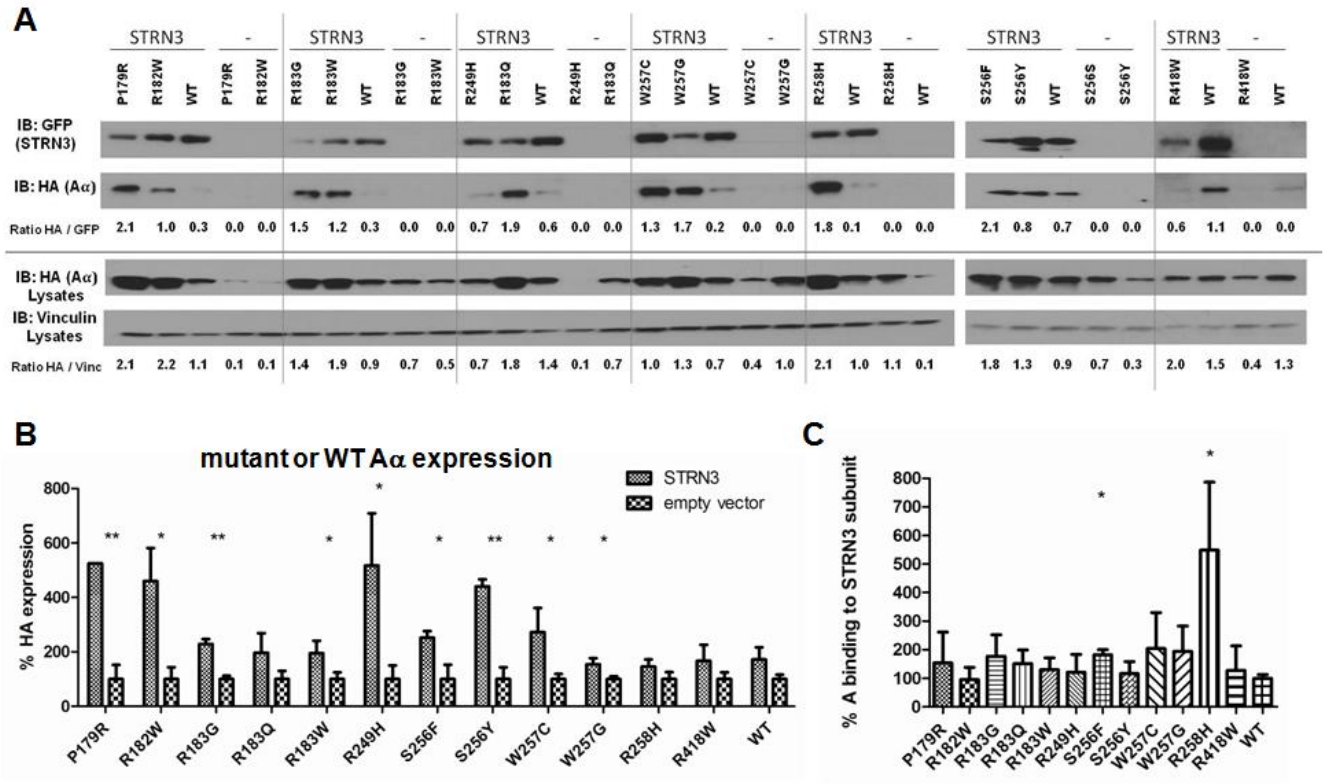


Figure 3: Binding of B‴ subunit STRN3 to endometrial cancer-related Aα mutants.
A. GFP-tagged STRN3 or GFP alone (-) were co-expressed with HA-tagged WT or mutant Aα in HEK293 cells. The HA signal in the complete lysates and in the isolated GFP-trapped complexes was determined by immunoblotting (IB). After quantification of the band intensities with Image J, ratios were determined between the GFP and HA signals in the GFP-trapped complexes, and between the vinculin and HA signals in the lysates. **B.** Mean values of quantified HA/vinculin ratios in the lysates, calculated relative to Aα WT control (set to 100%), from three independent experiments (*, $p < 0.05$; **, $p < 0.01$). **C.** Mean values of quantified HA/GFP ratios in the GFP-trapped complexes, calculated relative to Aα WT control, and further corrected for differences in expression with Aα WT control (panel **B**). Results are derived from three independent experiments (*, $p < 0.05$).

To provide further insights into the obviously different binding behavior of the Aα mutants to resp. the B, B′ and B″ subunits (unaltered, decreased or loss-of-binding), and the B‴/striatins (increased binding), we co-expressed HA-tagged WT Aα or Aα mutants (R182W, R183G/Q and S256F) with GFP-tagged B-type subunits (Bα, B′γ1, B′δ, B′ε, STRN3), and evaluated the presence of endogenous PP2Ac and the HA-tagged Aα subunits in the GFP-trapped complexes (*Figure 4*). Unfortunately, anti-A immunoblotting could not unambiguously reveal the presence of endogenous Aα because of insufficient resolution of the HA-tagged A and endogenous A bands on the SDS-PAGE gel (data not shown). Nevertheless, we found that the PP2Ac binding patterns in all cases completely mimicked those of the GFP-B-type subunits' inputs, while the anti-HA signals were completely different, but fully consistent with our other binding assays (*Figures 2 and 3*). Taken together, these data indicate that all B-type subunits likely bound a mixture of HA-tagged and endogenous A subunits. Only for STRN3, the fraction of bound mutant Aα was significantly higher than bound WT, providing additional proof for a gain-of-binding of STRN3 to the Aα mutants. B′δ bound comparable

amounts of mutant and WT Aα. B'γ1 and B'ε showed lower affinity to mutant Aα compared to WT, and Bα did not bind any mutant Aα at all.

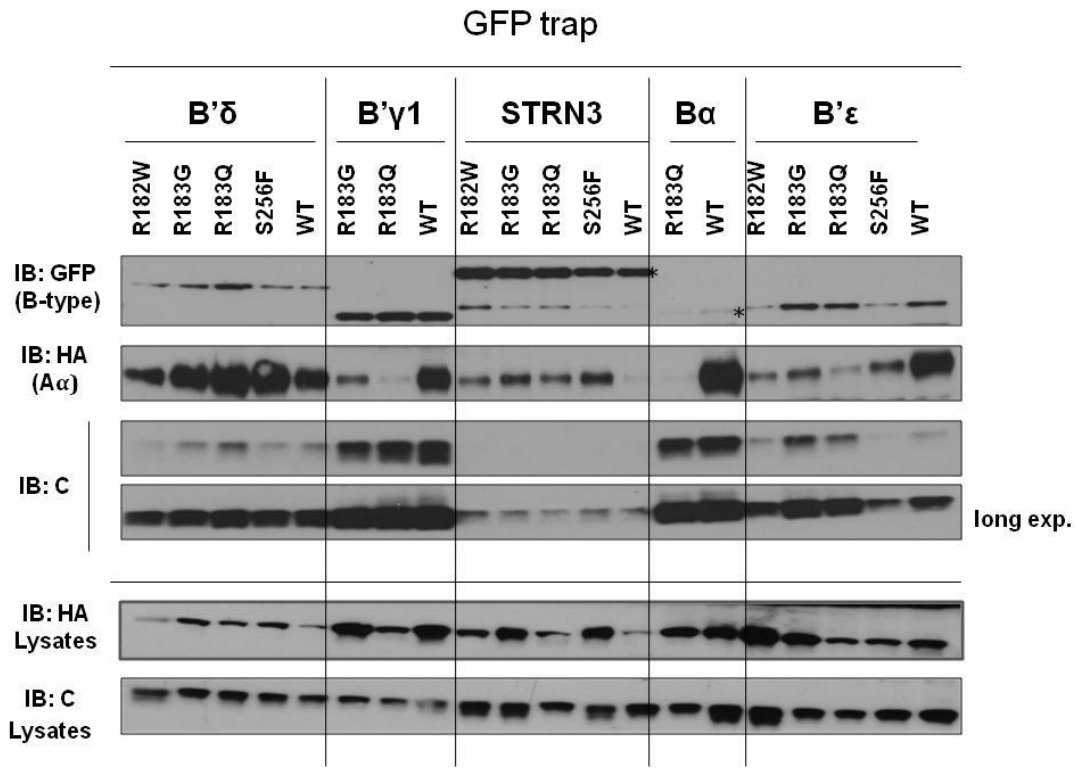


Figure 4: Comparative analysis of binding of Aα mutants and endogenous PP2Ac to GFP-tagged B-type subunits.
GFP-tagged Bα, B'γ, B'δ, B'ε and STRN3 were co-expressed with HA-tagged WT or mutant Aα in HEK293 cells. The presence of HA-tagged A variants and endogenous PP2Ac in the GFP-trapped complexes was determined by immunoblotting. For the anti-C immunoblot, two different exposures ('exp.') are shown. Asterisks indicate the GFP-STRN3 and GFP-Bα bands resp.

In summary, our binding assays in HEK293 cells revealed that cancer-associated Aα mutations differentially affect binding of Aα to different PP2A subunits.

Table 1. Summary of mutant Aα binding defects.

' ' : Aα mutant binding is *highly and significantly decreased* as compared to WT (<30%);

' ' : Aα mutant binding is *modestly, but significantly decreased* as compared to WT (>30%);

' ' : Aα mutant binding does *not significantly differ* from WT Aα;

' ' : Aα mutant is *significantly increased* as compared to WT Aα

'numbers' indicate percentage of residual Aα mutant binding compared to WT Aα (set at 100%)

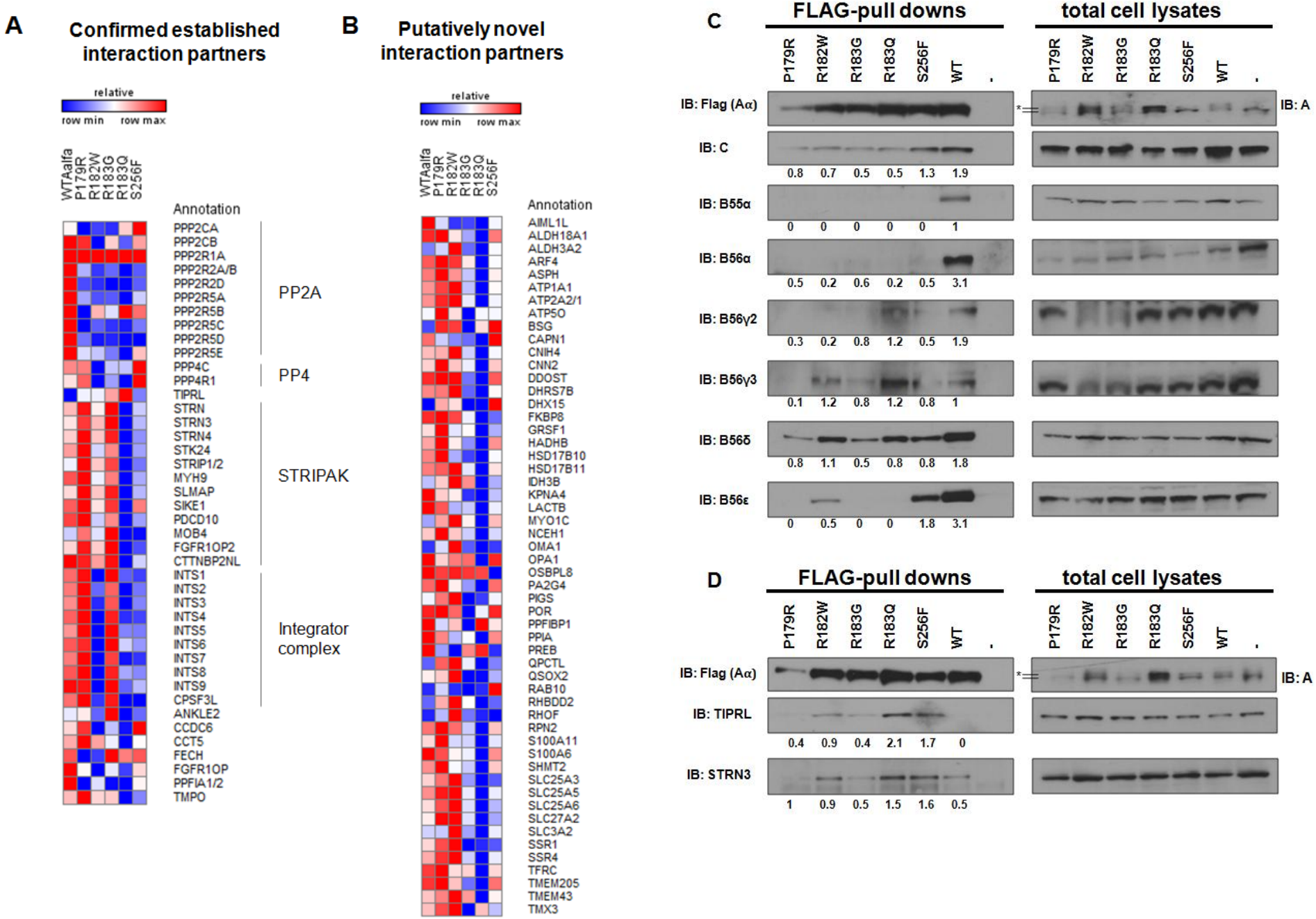
Mutation		C	B55α	B55β	B56α	B56β	B56γ1	B56δ	B56ε	B"α2 PR72	B"' STRN3
HR 5	P179R	11	11	0	13	0	3	50	33	55	154
	R182W	16	1	0	20	0	1	41	35	0	95
	R183G	39	3	0	48	0	46	64	43	111	177
	R183Q	31	10	0	83	0	65	135	93	4	152
	R183W	38	3	2	20	0	7	67	3	93	130
HR 7	R249H	131	177	73	155	79	97	117	106	116	121
	S256F	30	3	2	43	47	16	63	64	25	182
	S256Y	33	0	7	17	26	28	59	43	9	117
	W257C	39	2	3	31	9	20	89	98	97	204
	W257G	35	1	0	40	21	2	193	45	26	194
	R258H	32	0	0	12	0	12	57	14	12	549
R418W		22	22	1	33	34	20	47	37	33	127
Wild-type		100	100	100	100	100	100	100	100	100	100

Interactome of WT and mutant Aα in endometrial cancer cells

To validate and extend our targeted PP2A subunit binding assays to a pathological more relevant context, we expressed WT Aα and 5 selected Aα mutants (P179R, R182W, R183G, R183Q and S256F) as N-terminally FLAG-tagged proteins in the endometrial cancer cell line HEC-1-A by a lentiviral approach. HEC-1-A cells expressing FLAG peptide alone were used as a negative control. Following anti-FLAG pull down and subsequent elution by FLAG peptide, we analyzed the trapped complexes and their putative interaction partners by mass spectrometry (MS) in a semi-quantitative way. Besides validation of changes in the assembly

of the subset of PP2A holoenzymes expressed in endometrial epithelial cells, this approach also allowed identification of potential gain-of-function or loss-of-function alterations in the broader Aα interactome in an unbiased, non-targeted way.

Using this approach, we identified 96 cellular proteins as specific co-eluting (direct or indirect) interaction partners of Aα (*Figure 5*). 42 of these had previously been reported in PP2A interactome studies from HEK293 cells (64,65,66) (*Figure 5A*), while the remaining 54 are potentially novel (*Figure 5B*). Details on the MS data, quantifications and normalizations can be found in *Suppl. Data S3 (Excel file)*. The known interaction partners include 12 established PP2A subunits (Cα, Cβ, R2A/Bα, R2D/Bδ, R5A/B'α, R5A/B'β, R5C/B'γ, R5D/B'δ, R5E/B'ε, the B''' subunits STRN, STRN3 and STRN4), 2 PP4 subunits (C and R1), a reported cellular PP2A inhibitor (67) (TIPRL1), a number of well-established B-type subunit interactors (nine components of the STRIPAK complex (64,65,68), the B'γ/B'δ interactors PPFIA1/2 (liprin α1/2) (69,70)), and a number of poorer characterized (indirect) Aα binding partners (ten subunits of the Integrator complex, ANKLE2, CCDC6, CCT5, FECH, FGFR1OP and TMPO). Overall, the R183Q and S256F mutants show the most pronounced binding deficiencies to several of the interactors identified, followed by the R183G and R182W mutants, and finally, the P179R mutant which shows the least binding deficiencies when compared to WT Aα. Notably, the MS data revealed that the Aα mutants showed lower binding affinity for the PP2A C, B and B' subunits, suggesting that these mutations are loss-of-function (*Figure 5A*). This was further confirmed by direct immunoblotting of the anti-FLAG pull downs with available isoform-specific PP2A subunit antibodies (*Figure 5C*). However, these immunblots also revealed sustained binding of specific Aα mutants to specific B' subunits (*Figure 5C*), consistent with our binding assays in HEK293 cells (*Figure 2*). In contrast, the MS data suggested either largely unaltered, or increased binding to the Aα mutants for the PP2A B'''/STRN subunits, rather indicating gain-of-function effects of these Aα mutations (*Figure 5A*). This result was also validated by direct immunoblotting (*Figure 5D*). Thus, the (mutant) Aα interactome data from HEC-1-A cells largely confirm results from our binding assays in HEK293 cells with ectopically expressed Aα and B-type subunits (*Table 1*). The reliability of the MS data is further underscored by the observation that interactors belonging to one and the same multi-protein complex (e.g. STRIPAK, Integrator) show an identical differential binding behavior to the different Aα mutants. Finally, the MS data revealed the most obvious and strongest gain-of-function effect of the Aα mutants for the PP2A inhibitor TIPRL1 (*Figure 5A*). We validated the enhanced interaction of Aα mutants to TIPRL1 by immunoprecipitation and TIPRL1 immunoblotting (*Figure 5D*). Taken together, we observed bidirectional effects of Aα mutations on the formation of specific complexes, making it challenging to define their mode of action as loss-of-function or gain-of-function.


A
Confirmed established interaction partners
relative
row min
row max
WTAalfa
P179R
R182W
R183G
R183Q
S256F
Annotation
PPP2CA
PPP2CB
PPP2R1A
PPP2R2A/B
PPP2R2D
PPP2R5A
PPP2R5B
PPP2R5C
PPP2R5D
PPP2R5E
PPP4C
PPP4R1
TIPRL
STRN
STRN3
STRN4
STK24
STRIP1/2
MYH9
SLMAP
SIKE1
PDCD10
MOB4
FGFR1OP2
CTTNBP2NL
INTS1
INTS2
INTS3
INTS4
INTS5
INTS6
INTS7
INTS8
INTS9
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ANKLE2
CCDC6
CCT5
FECH
FGFR1OP
PPFIA1/2
TMPO
PP2A
PP4
STRIPAK
Integrator complex
B
Putatively novel interaction partners
relative
row min
row max
WTAalfa
P179R
R182W
R183G
R183Q
S256F
Annotation
AIML1L
ALDH18A1
ALDH3A2
ARF4
ASPH
ATP1A1
ATP2A2/1
ATP5O
BSG
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CNIH4
CNN2
DDOST
DHRS7B
DHX15
FKBP8
GRSF1
HADHB
HSD17B10
HSD17B11
IDH3B
KPNA4
LACTB
MYO1C
NCEH1
OMA1
OPA1
OSBPL8
PA2G4
PIGS
POR
PPFIBP1
PPIA
PREB
QPCTL
QSOX2
RAB10
RHBDD2
RHOF
RPN2
S100A11
S100A6
SHMT2
SLC25A3
SLC25A5
SLC25A6
SLC27A2
SLC3A2
SSR1
SSR4
TFRC
TMEM205
TMEM43
TMX3
C
FLAG-pull downs
total cell lysates
P179R
R182W
R183G
R183Q
S256F
WT
-
IB: Flag (Aα)
IB: A
IB: C
0.8 0.7 0.5 0.5 1.3 1.9
IB: B55α
0 0 0 0 0 1
IB: B56α
0.5 0.2 0.6 0.2 0.5 3.1
IB: B56γ2
0.3 0.2 0.8 1.2 0.5 1.9
IB: B56γ3
0.1 1.2 0.8 1.2 0.8 1
IB: B56δ
0.8 1.1 0.5 0.8 0.8 1.8
IB: B56ε
0 0.5 0 0 1.8 3.1
D
FLAG-pull downs
total cell lysates
P179R
R182W
R183G
R183Q
S256F
WT
-
IB: Flag (Aα)
IB: A
IB: TIPRL
0.4 0.9 0.4 2.1 1.7 0
IB: STRN3
1 0.9 0.5 1.5 1.6 0.5

Figure 5: Mass spectrometry-based identification of WT and mutant Aα interactomes in HEC-1-A endometrial cancer cells.

A,B. Semi-quantitative heat maps of WT and mutant Aα interactomes in HEC-1-A cells. Putative Aα interacting proteins were identified by mass spectrometry in FLAG peptide eluates of FLAG pull down assays, performed on lysates of HEC-1-A cells stably expressing N-terminally FLAG-tagged Aα variants, or FLAG-tag alone. To be scored as a potentially genuine interaction partner, the overall peptide abundance of a given protein should be at least 3 times as abundant as in the FLAG alone control. This resulted in 96 putative Aα interaction partners. Heat maps display the relative abundance of each interaction partner in the different Aα pull downs: regardless of absolute abundances, the interactor occurring in the highest abundance is given a dark red color and the lowest abundant partner is given a dark blue color. Data interpretation, therefore, needs to be done with caution, preferably with the list of absolute abundances at hand (Excel file Suppl. Data S3), as small differences in abundance can be similarly displayed as marked differences in abundance. Panel **A** displays previously reported PP2A interactome constituents; panel **B** displays putatively novel Aα interactors. **C.** Validation of endogenous PP2Ac binding and of several endogenous PP2A B and B' subunits to mutant Aα. The presence of the indicated PP2A subunits in anti-FLAG pull downs (left) and total cell lysates (right) of the transduced HEC-1-A cells was determined by immunoblotting (IB) with available isoform-specific antibodies. * indicates FLAG-tagged Aα in the lysates. Quantifications represent the calculated ratios between the IB:C or B signals and the IB:FLAG signal for a given Aα mutant in the anti-FLAG pull downs. **D.** Validation of gain of TIPRL1 and STRN3 binding to mutant Aα. Same approach as in panel **C** to determine endogenous TIPRL1 and STRN3 levels in anti-FLAG (mutant) Aα pull downs.

Cancer-associated Aα mutants increase anchorage-independent growth and xenograft growth *in vivo* of endometrial carcinoma cells

We next analyzed whether ectopic expression of the mutant Aα proteins in HEC-1-A cells affected the oncogenic properties of these endometrial cancer cells. First, we confirmed that both *PPP2R1A* alleles in HEC-1-A cells are wildtype by Sanger sequencing (data not shown). The growth-inducing potential of the Aα mutants was tested in soft agar assays *in vitro* and xenograft assays in immune-compromised mice (*Figure 6*). These functional studies revealed that all Aα mutants tested show an increased tumorigenic potential *vs*. WT Aα upon their expression in HEC-1-A cells. This is particularly clear for R183G, R183Q and S256F since the size and number of colonies grown in soft agar (*Figure 6A*) and the number and the size of xenografted tumors in mice (*Figure 6B*) was significantly increased compared to WT Aα expressing cells and negative control (parental HEC-1-A cells). The effect for P179R and R182W mutants was weaker (*Figure 6A,B*), although for P179R this might be related to its markedly lower expression levels (*Figure 5C*).

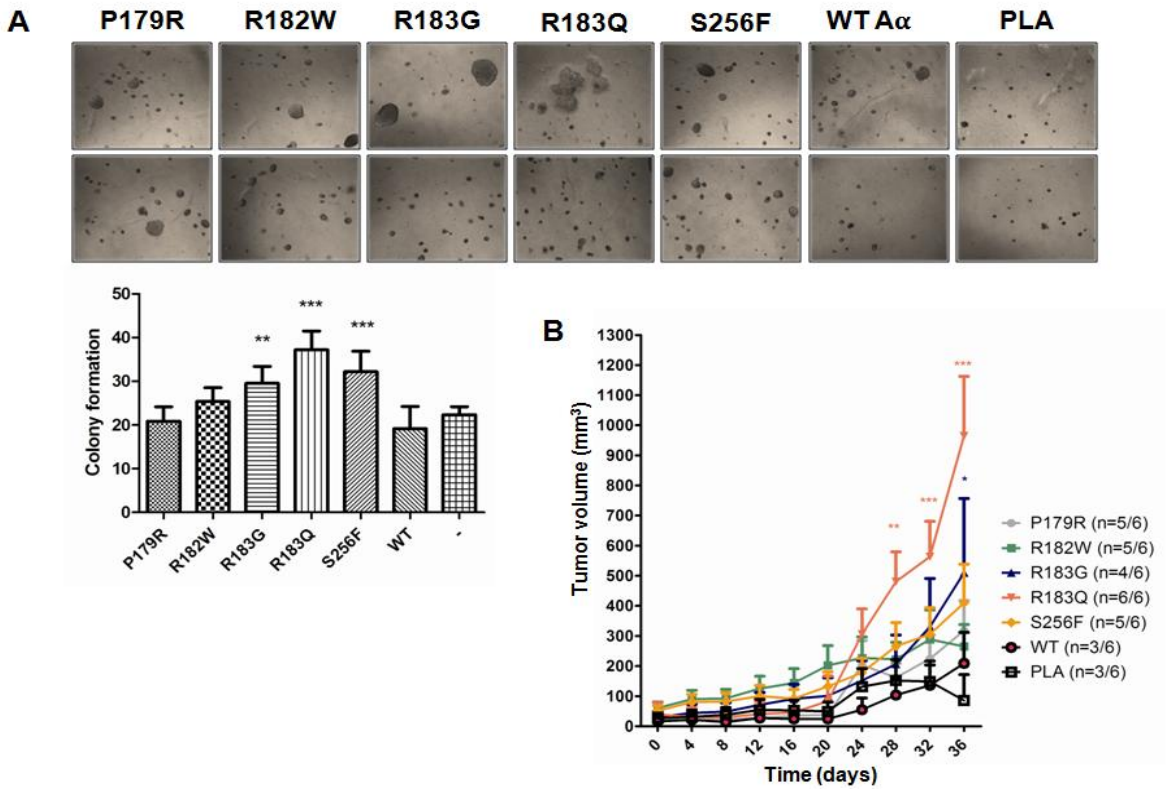

Figure 6: Tumorigenic phenotypes of endometrial cancer cells expressing Aα mutants.
A. Anchorage-independent growth of HEC-1-A cells, ectopically expressing mutant or WT Aα subunit. The pictures give an idea about the number and size of colonies obtained for each condition at a given time point after seeding. The data in the graph represent the mean (±SD) of the colony number from 3 replicates and 6 measurements. Data were analyzed via the ANOVA test (**p < 0.01; *** p < 0.001). **B.** Tumor growth of xenografted HEC-1-A cells. Size (graph) and number of tumors (n) are indicated for each condition (n=6). Differences in tumor size were analyzed via the ANOVA test (* p < 0.05; ** p < 0.01; *** p < 0.001).

Cancer-associated Aα mutants promote hyperphosphorylation of the Akt and mTOR/p70S6K signaling pathways and decrease phosphorylation of the MAPK pathway

To determine the effects of mutant or WT Aα expression on oncogenic signaling, we performed immunoblotting analysis using antibodies directed against several established cancer-related PP2A substrates and components of cancer-associated signaling pathways (*Figure 7*). These experiments revealed significantly increased oncogenic signaling in cells expressing the strong tumorigenic mutants R183G/Q and S256F, as demonstrated by hyperphosphorylation of phospho-p70S6 kinase and phospho-S6 (*Figure 7A,D*), phospho-GSK3β and phospho-Akt T308 (*Figure 7B,D*). For the P179R and R182W mutants, an effect on only phospho-GSK3β was seen (*Figure 7B,D*), consistent with their milder tumorigenic phenotype in the functional assays (*Figure 6*). Remarkably, we found that activity/phosphorylation of ERK1/2 was decreased in all cases (*Figure 7C,D*). These results suggest that increased tumorigenic potential induced by Aα mutants could be triggered by up-regulation of the Akt, GSK-3β and mTOR signaling pathways.

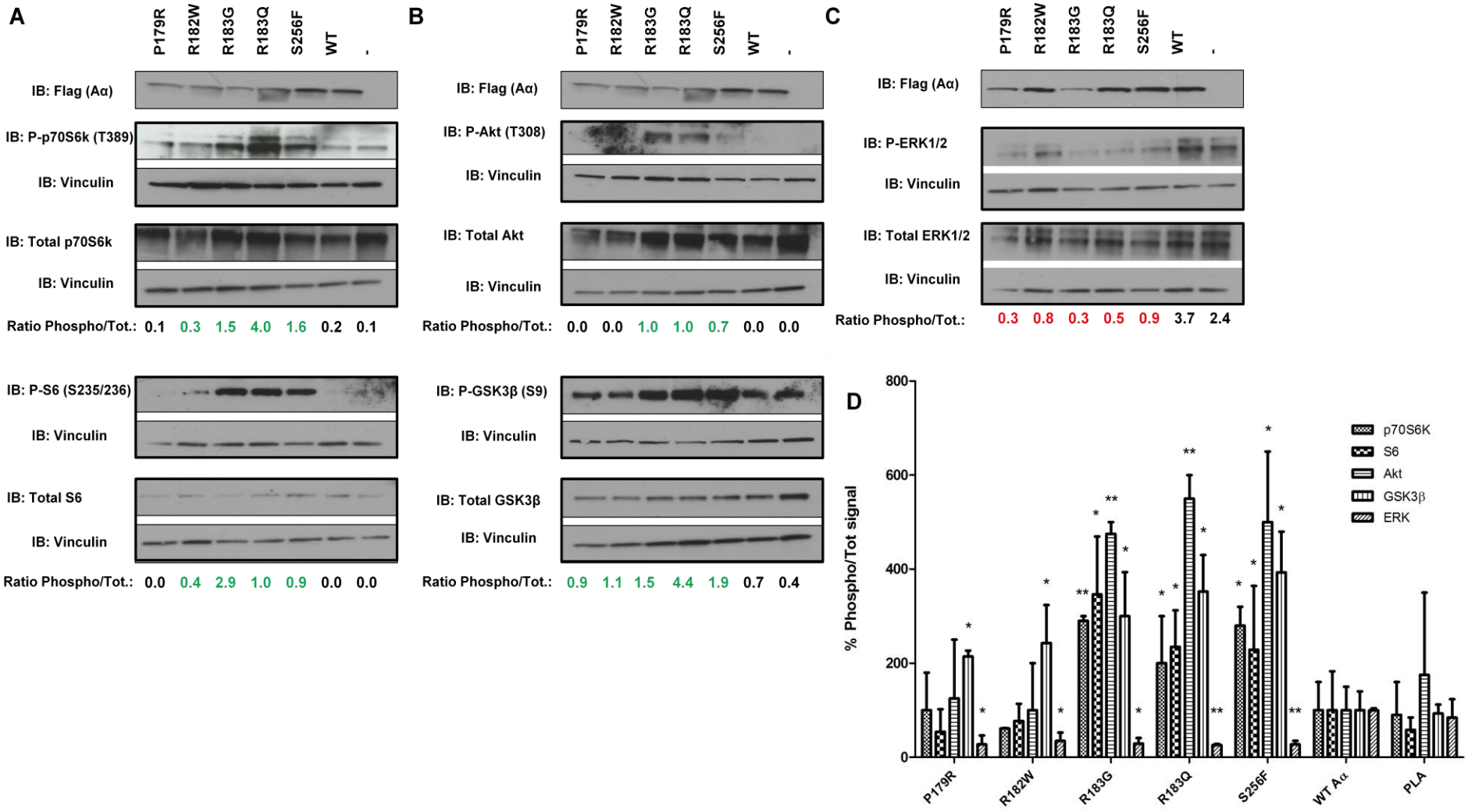

Figure 7: Effects of mutant Aα expression on oncogenic signaling pathways in HEC-1-A cells.

Cell lysates of WT or mutant Aα expressing HEC-1-A cells were subjected to immunoblotting (IB) with the indicated antibodies and signals were quantified using ImageJ. 'Total' and 'Phospho' signals were determined on different blots, which were both redeveloped for vinculin to correct for loading differences. Differences with WT are indicated in color: green for an increase as compared with WT; red for a decrease as compared with WT. Representative images of one of three independent (i.e. three different lentiviral transductions) experiments are shown (**A**,**B**,**C**). **D**. Mean values of quantified Phospho/Tot ratios in the lysates, calculated relative to Aα WT control (set to 100%) from three independent experiments (ANOVA test; *, $p < 0.05$; **, $p < 0.01$).

Mutant Aα proteins act in a dominant manner through formation of substrate-trapping, catalytically deficient AB'C complexes

Since we observed increased oncogenicity upon mutant Aα expression, without prior reduction of endogenous Aα in HEC-1-A cells (*Figures 6 and 7*), it seemed very unlikely that the tested cancer-associated Aα mutants acted by a mechanism of haploinsufficiency. In addition, we did not observe a clear correlation between the number of B-type subunit binding deficiencies for a given mutant (*Table 1*, *Figure 2B, Figure 3, Figure 5C,D*) and the degree of their oncogenic behavior in tumor growth (*Figure 6*) or oncogenic signaling (*Figure 7*). On the other hand, such correlation was more obvious for the observed gain-of-binding of the Aα mutants to STRN3 and TIPRL1 (*Figure 3, Figure 5D*), although it was still not entirely consistent (e.g. compare R183G with R182W or P179R). Particularly, a gain of function of PP2A-STRN is difficult to reconcile with decreased dephosphorylation of the oncogenic kinases GSK3β, Akt and p70 S6K (*Figure 7A,B*), which are actually reported substrates of PP2A-B'β, B'γ and B'δ isoforms (42,45,71,72,73,74). On the other hand, ERK phosphorylation could be negatively regulated by PP2A-STRN through inactivation/dephosphorylation of Mst kinases (75,76,77,78), and this might explain the observed decrease in ERK phosphorylation (*Figure 7C*), but not the increase in cancer cell growth. Therefore, our data eventually emerged in the hypothesis that the Aα mutants might exert their tumorigenic effect through a dominant-negative mechanism: by retaining binding to certain B-type subunits, in particular to B'β, B'γ and B'δ, the Aα mutants might form substrate trapping complexes with decreased PP2A activity that are capable to compete with active, WT Aα-containing PP2A-B'β/γ/δ trimers for substrate binding. Indeed, as overall PP2Ac binding to the Aα mutants was decreased (*Figure 1*), these dominant complexes might be mutant A-B complexes devoid of C subunit, or alternatively, as the PP2A inhibitor TIPRL1 showed marked gain-of-binding to all Aα mutants (*Figure 5A,D*), these dominant complexes might be mutant A-B-C holoenzymes with catalytically impaired C subunit through increased recruitment of TIPRL1.

To experimentally test whether B-type subunits can form A-B complexes without C, we first expressed an HA-tagged Aα deletion mutant (Aα aa1-410), lacking the PP2Ac binding domain (HEAT-repeats 11 to 15) in HEK293 cells, and determined its binding to several GST-tagged Btype subunits (B, B' and B") and GFP-tagged STRN3 (B'") (*Figure 8*). While anti-PP2Ac immunoblots of HA pull downs confirmed that Aα aa1-410 does indeed no longer bind PP2Ac (*Figure 8A*), GST pull down and GFP trapping experiments show that only B'γ1, B'δ, PR72 and STRN3 retain significant binding to Aα aa1-410, while Bα, Bβ, B'α and B'ε do not (*Figure 8B,C*). These findings further contribute to the notion that remarkable differences exist between different B-type subunits and subunit isoforms in their binding behavior to the A subunit, and that specific B-type subunits may indeed form A-B complexes without C.

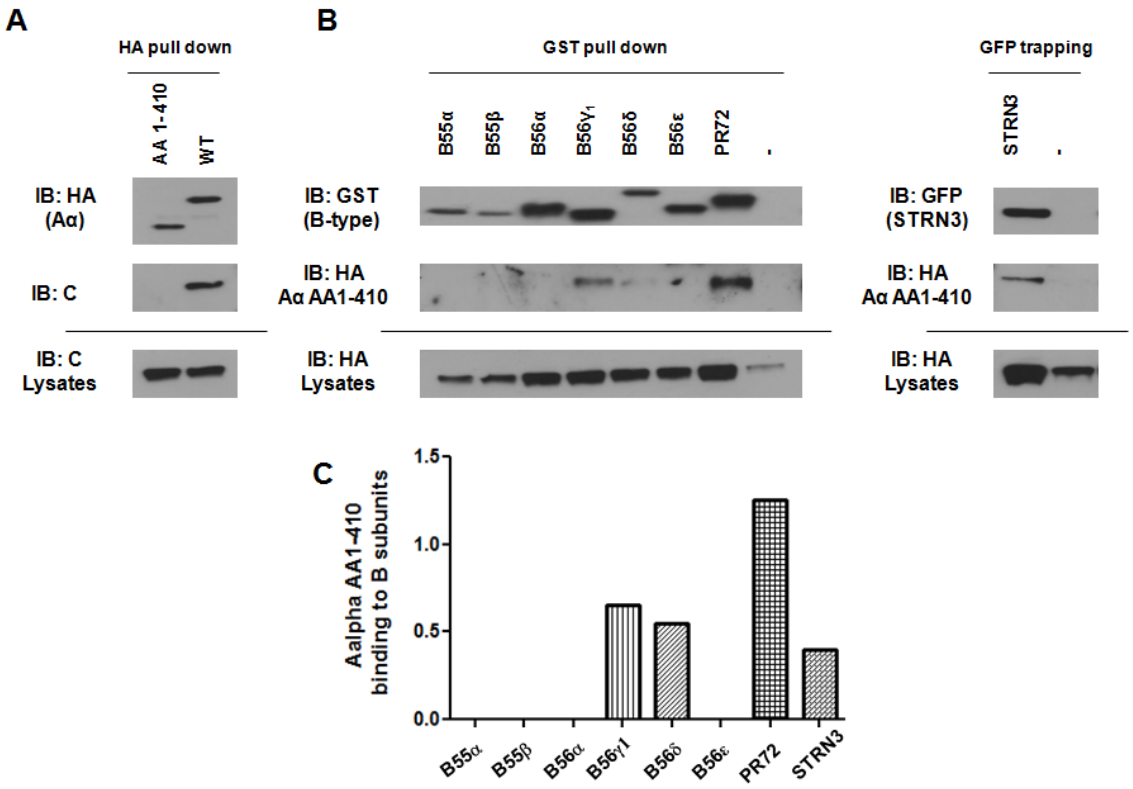

Figure 8: Select B-type subunits can bind Aα independently of Aα–C interaction.
Multiple GST-tagged representatives from the B, B′ and B″ subunit families, or GFP-tagged STRN3 (B‴) were co-transfected in HEK293 cells with the Aα deletion mutant containing HEAT repeats 1-10 (amino acids 1-410) and thus lacking the PP2Ac binding domain. **A.** Lack of PP2Ac binding to Aα aa1-410. The presence of PP2Ac in HA pull downs from HEK293 cells expressing HA-tagged Aα aa1-410 was determined by immunoblotting (IB). **B.** Binding characteristics of several PP2A B-type subunits to HA-tagged Aα aa1-410. Upon GST pull down (left) or GFP trapping (right), interaction with the Aα deletion mutant was examined by immunoblotting (IB) with anti-HA antibody. **C.** After quantification of the band intensities with Image J, ratios were determined between the GST/GFP and HA signals and calculated relative to Aα WT control (set to 100% for each B-type subunit pull down). Mean values of two independent experiments are shown.

To further substantiate these findings in isolated mutant A-B complexes, we performed serial pull downs ("IP-on-IP") on GFP-TEV-B′δ and several HA-tagged Aα mutants, and compared their ability to bind C subunit with the WT A-B′δ complex (*Figure 9*). Apparently, all mutant Aα-B′δ tested (R183G, R183Q and S256F) still bound PP2Ac in comparable amounts as WT Aα-B′δ (*Figure 9A*, left, ratio C/HA). When the same experiment was repeated in the presence of ectopically expressed TIPRL1, relatively more PP2Ac was retrieved in the mutant Aα-B′δ complexes than in WT Aα-B′δ (*Figure 9A*, right). Moreover, TIPRL1 itself was also present in the trimeric complexes (*Figure 9A*). Interestingly, when phosphatase activities in the retrieved complexes was measured, specific PP2Ac activity was found to be decreased in the mutant Aα-B′δ-C complexes when TIPRL1 was co-expressed with GFP-TEV-B′δ and HA-Aα (*Figure 9B*).

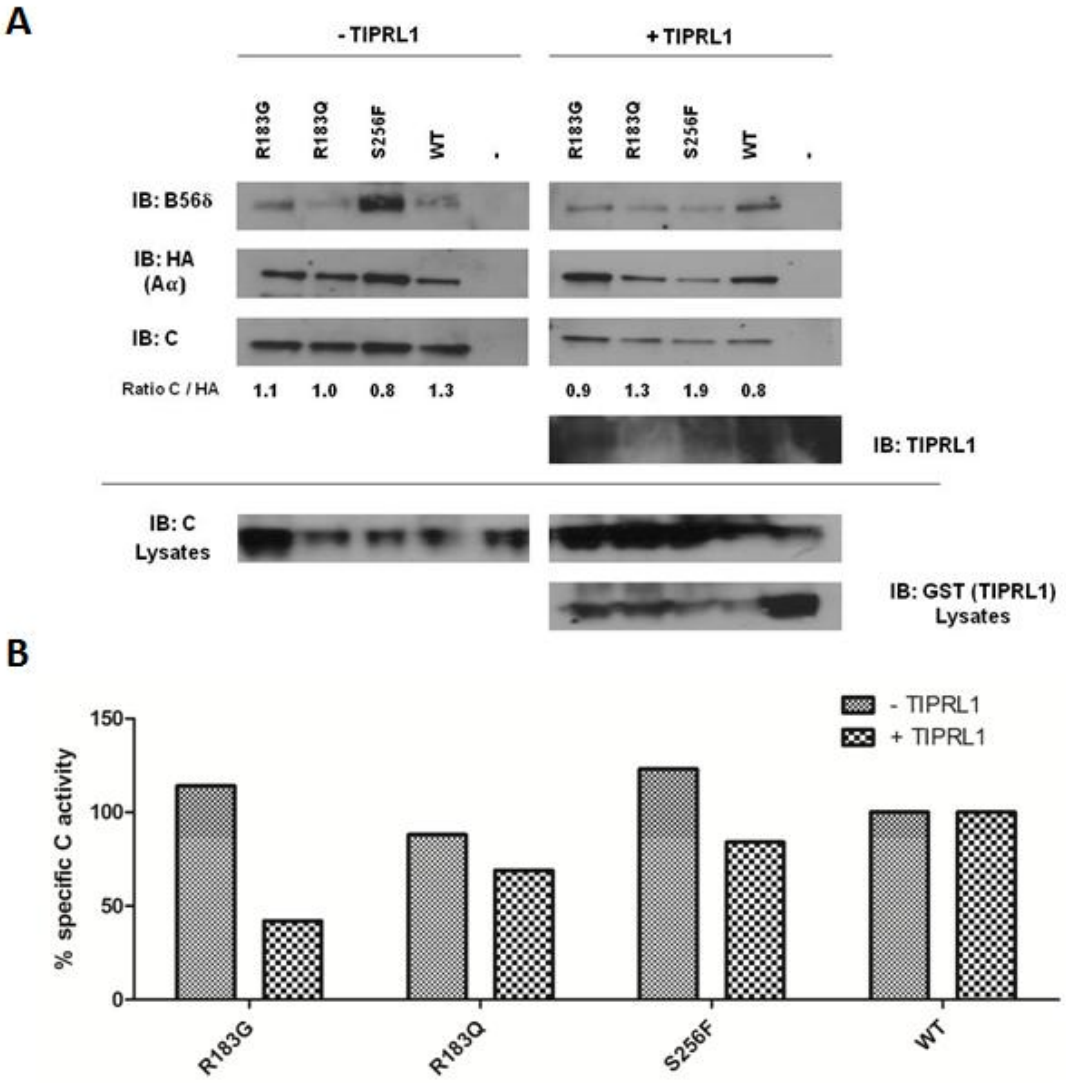

Figure 9: Biochemical characterization of mutant Aα-B′δ complexes.
EGFP-TEV-B′δ was co-expressed with HA-tagged WT or mutant Aα, with or without GST-tagged TIPRL1. **A.** Following serial pull down ("IP-on-IP", details in materials and methods), the presence of PP2Ac and TIPRL1 in the Aα-B′δ complexes was analyzed by immunoblotting (IB) with PP2Ac or TIPRL1 antibodies. After quantification of the band intensities with Image J, the ratios between C and HA signals were determined and calculated relative to Aα WT (set to 100%). **B.** PP2A activity measurements in isolated Aα-B′δ complexes (IP-on-IP). For activity measurements, the pmole number of released phosphate from the R-R-A-pT-V-A phosphopeptide (750µM in assay) was determined by Malachite Green assay. For calculation of specific PP2A activities, each activity measurement was corrected for the amount of C present in the isolated complexes, determined via immunoblotting (IB) and quantified by ImageJ. All specific activities were eventually recalculated relative to Aα WT (set to 100%).

To provide further insights into the inhibitory effects of TIPRL1 on different PP2A complexes, we purified recombinant TIPRL1 from bacteria (*Figure 10A*), and assayed its effect on isolated PP2A complexes of different composition *in vitro*. We observed the strongest inhibitory effect on the isolated PP2A core dimer (*Figure 10B*), while, at the same concentration, TIPRL1 failed to show any discernable inhibitory effects on isolated B'γ1- and B'δ-containing PP2A trimers (*Figure 10C*). Remarkably, however, when the same trimers,

now harboring a mutant Aα protein (R183Q or R183G) instead of WT Aα were assayed in the presence of TIPRL1, a clear inhibition of PP2A activity could be observed (*Figure 10D,E*). Thus, our data reveal that Aα mutation, through increased affinity for the PP2A inhibitor TIPRL1, suppresses phosphatase activity of specific PP2A trimers. These catalytically impaired trimers that, despite Aα mutation, can still be assembled and that are characterized by the presence of a specific B-type subunit, may subsequently interfere with normal dephosphorylation by WT Aα-containing trimers, harboring the same B-type subunit, through a competition mechanism for binding to B-type subunit targeted substrates. This clearly explains their ability to increase cancer cell growth and oncogenic signaling upon their ectopic expression in HEC-1-A cells harboring two WT *PPP2R1A* alleles, and underscores their contribution to endometrial cancer development by a dominant-negative mechanism-of-action.

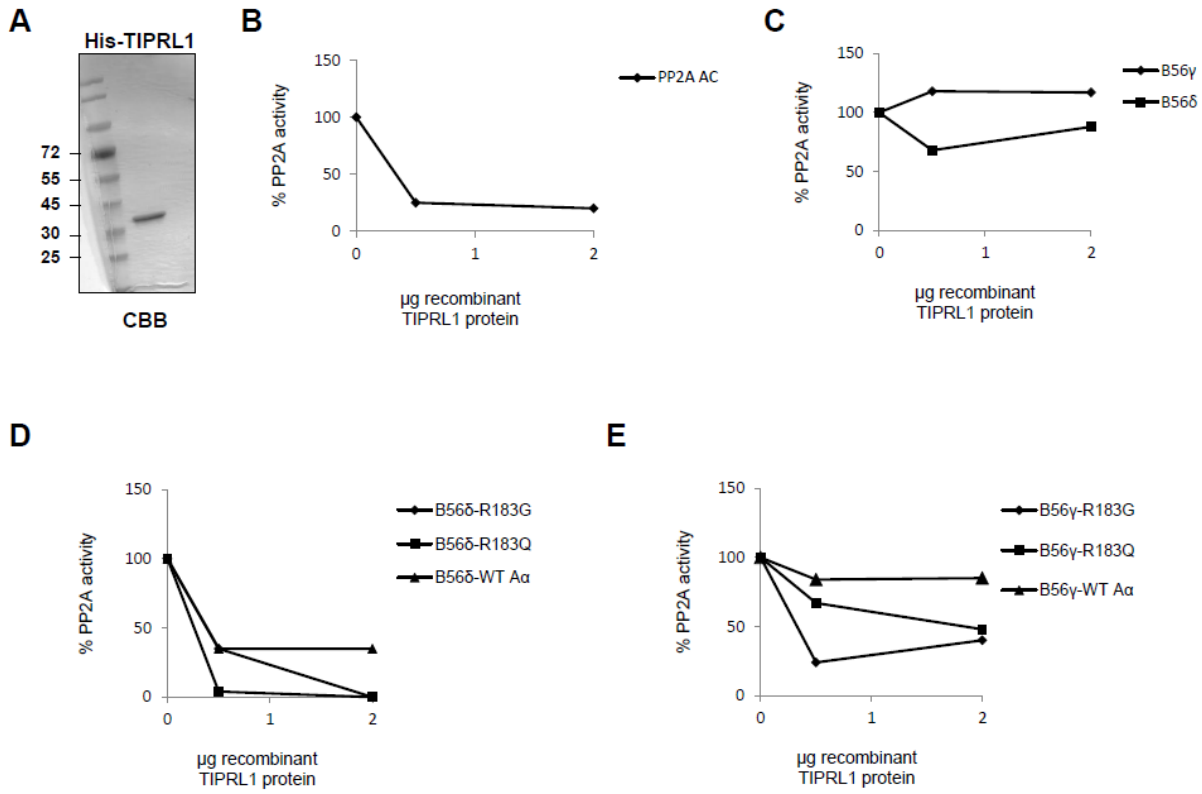

Figure 10: Effects of recombinant TIPRL1 on different PP2A complexes
A. Coomassie Brilliant Blue (CBB) stained SDS-PAGE gel of His-TIPRL1, purified from *E. coli*. **B.** TIPRL1 efficiently inhibits the PP2A core dimer. *De novo* purified PP2AD (Millipore) (1/80 in 20µl enzyme dilution buffer) was pre-incubated for 15' at 30°C with 20µl enzyme dilution buffer alone, or with 0.5 µg or 2 µg recombinant TIPRL1 in 20µl enzyme dilution buffer. PP2A activity was determined on R-R-A-pT-V-A phosphopeptide, and calculated relative to the condition without addition of TIPRL1 (set at 100%). **C.** Same experiment as in panel B, but instead of PP2A dimer, PP2A-B56γ1 and PP2A-B56δ trimers, isolated from GFP-B56γ1 and GFP-B56δ expressing HEK293 cells by GFP-trapping, were used. **D-E.** Same experiment as in panel B with PP2A-B56δ trimers harboring HA-tagged WT or mutant Aα, isolated by 'IP-on-IP' approach from GFP-TEV-B56δ expressing HEK293 cells (**D.**), or with PP2A-B56γ1 trimers harboring HA-tagged WT or mutant Aα, isolated by 'IP-on-IP' from HEK293 cells expressing GFP-TEVB56γ1 (**E.**).

DISCUSSION

Heterozygous missense mutation of *PPP2R1A* appears to be a recurrent event in human cancer. There is a prevailing view that *PPP2R1A* mutations are loss-of-function due to impairment of PP2A holoenzyme formation. This can be either due to loss of PP2Ac binding and reduced binding to all B-type subunits (R418W) (45,55,63), or to decreased or loss of binding to all B' subunits (E64D/G) (45,55,57,63). This results in haploinsufficieny of Aα, primarily affecting holoenzyme formation with B-type subunits which no longer bind to mutant Aα and show the least binding affinity for WT Aα, and therefore, lose the competition with other B-type subunits to bind with the remaining 50% WT Aα and form PP2A trimers. When tested in HEK293 cells, particularly formation of PP2A-B'γ trimers becomes significantly impaired under these circumstances, resulting in Akt hyperphosphorylation and increased tumor growth (45). However, our current results strongly indicate that this model, which is based on the characterization of a few, sporadically occurring Aα mutants, cannot be generalized. Indeed, we found that 10 of 11 recurrently occurring endometrial cancer-associated Aα mutants affect PP2A activity by a completely different mechanism. All these heterozygous mutants harbor a single amino acid substitution clustering in HEAT-repeat 5 (P179L, P179R, R182W, R183G, R183Q, R183W) or HEAT-repeat 7 (R249H, S256F, S256Y, W257C, W257G, R258H). Such clustering and the recurrence of these mutations, not only in endometrial cancers patients, but sporadically also in patients of other cancer types, represent strong genetic evidence for a dominant-negative or gain-of-function mechanism-of-action, rather than haploinsufficiency.

Our binding assays with ectopically expressed Aα mutants and B-type subunits in HEK293 cells, as well as with ectopically expressed Aα mutants and endogenous B-type subunits in HEC-1-A cells, showed an unexpectedly complicated picture of diverse binding defects to 10/11 mutants tested. Only Aα mutant R249H largely behaved like WT, and may therefore be a nonpathological passenger mutant. Published crystallographic data of different PP2A trimers support this view, revealing that the R249 residue is buried inside the protein, while all other mutated residues face the B and C subunits (27). All other Aα mutants showed loss of binding to B55 subunits (isoforms α and β in HEK293 cells; isoforms α and δ in HEC-1-A cells); thus, a single missense mutation in either HR5 or HR7 suffices to severely affect binding to probably all isoforms of this PP2A subunit class. For the B56 and B" subunits, a completely different picture emerged. In this case, the binding deficiencies were much more diverse. Only one subunit, B56δ, still appeared to bind all Aα mutants tested in comparable amounts as WT, both in HEK293 and HEC-1-A cells. In contrast, for the B"'/striatin family members, our binding data revealed for the first time a gain-of-binding to Aα mutant proteins, demonstrated again in both cell models. Increased binding of the striatins to the Aα mutants may in part be an indirect consequence of their generally decreased binding to subunits of other subclasses (B55, B56 and B"). STRN3 may also have a stabilizing effect on the mutants, because they bind better.

We also found a general decrease in PP2Ac binding to 10/11 Aα mutants. In accordance, Aα deletion mutants of HR 5 and 7 were previously reported to show reduced binding to the C subunit (21). This highlights a complex relationship between B and C subunits within the holoenzyme: not only B subunits may require stabilizing contacts with PP2Ac to bind A and

form stable PP2A trimers (22,30,32,33,34), also the reverse can be true, i.e. stable PP2Ac binding to A can be dependent on the presence of the B subunit. Therefore, we presume that the overall reduced binding of PP2Ac to the Aα mutants, is an indirect consequence of the reduced or loss of binding of specific B-type subunits. However, some B-type subunits, in particular, B'γ1, B'δ, PR72 and STRN3, can form stable A-B complexes without C, as illustrated by their binding to Aα aa1-410, lacking the PP2Ac binding HEAT-repeats 11 to 15. This finding is consistent with earlier data, demonstrating that neither B'δ, PR72 or the striatins require any contacts with the six carboxyterminal residues of the PP2Ac tail to assemble into a PP2A holoenzyme, while all other B' subunits (α,β, ε isoforms) clearly do (B'γ was not tested) (33,35), and with recent structural data on formation of a stable A-B" dimer (31). Despite these findings, our subsequent IP-on-IP data with B'δ did not support formation of mutant A-B'δ complexes devoid of PP2Ac for any of the Aα mutants tested (R183G/Q, S256F).

Instead, an unbiased MS-based approach to identify differences in mutant Aα binding to constituents of the broader Aα interactome in HEC-1-A cells, unexpectedly, revealed TIPRL1, a previously reported cellular PP2A inhibitor (67), as the only cellular protein that showed significant gain-of-binding to all Aα mutants tested (P179R, R182W, R183G, R183Q, S256F). Subsequently, we showed that TIPRL1 co-expression or addition of recombinant TIPRL1 *in vitro*, resulted in decreased PP2A activity in isolated mutant Aα-B'δ-C complexes when compared with WT Aα-B'δ-C complexes, suggesting that increased TIPRL1 binding could result in the formation of catalytically impaired, mutant Aα containing PP2A trimers, which might act as dominant-negatives towards WT Aα containing trimers. The dominant-negative nature of the mutants was significantly further underscored by increased cell and tumor growth, and corresponding increased oncogenic signaling (hyperphosphorylation of p70S6K and S6, Akt T308, GSK3β) upon their ectopic expression in HEC-1-A cells, without any prior downregulation of endogenous Aα. This definitely argues against a mechanism of haploinsuffciency, and virtually eliminates the putative involvement of the observed loss-of-binding to specific B-type subunits (e.g. to B55 and others) as a major cause of the observed phenotype. Expression of R183G, R183Q and S256F resulted in a more severe phenotype than expression of P179R and R182W. For the R182W mutant, the number of tumors in the mice was higher compared to WT Aα, and especially in the early days after injection, the tumors grew faster, although the difference did not reach statistical significance. The soft agar assay showed a slight, but statistically non-significant, elevated number of colonies. Xenograft assays with P179R expressing HEC-1-A cells resulted in the formation of more tumors compared to WT Aα expressing cells, without any discernable difference in size. We suspect however, that the minimally increased tumorigenic potential of the P179R mutant may in part be due to its poorer expression following lentiviral transduction. The functional differences between the mutants were reflected well at the signaling level: hyperphosphorylation of p70S6K (and S6), Akt T308 and GSK3β S9 was clearly seen for R183G, R183Q and S256F and of GSK3β for R182W and P179R. Hyperactivation of these pathways indeed stimulates protein translation, cell growth/proliferation and survival. Although all mutants showed increased binding to STRN3, a gain-of-function of PP2A-STRN3 complexes cannot explain the observed hyperphosphorylation of the above oncogenic kinases. Instead, Akt, GSK-3β and p70S6K are reported substrates of PP2A-B' complexes in

several cellular contexts, particularly of B'β, B'γ- and B'δ-containing PP2A trimers (42,45,71,72,73,74,79). Accordingly, in HEC-1-A cells, the R183Q mutant, causing the most severe tumor phenotype, retains binding to B'γ2, B'γ3 and B'δ, and based on the MS data, also to B'β. This mutant also shows the strongest interaction with TIPRL1. For the other mutants, a similar reasoning could be applied, whereby the severity of the observed growth phenotype may eventually be the combinatory result of (1) the specific kind of B' subunit that still retains binding, (2) the number of B' subunits that retain binding, (3) the absolute strength of the retained binding (or binding affinity) to a specific B' subunit, and finally, (4) the binding efficiency to TIPRL1. Notably, the more B' subunits retain binding to an Aα mutant to form different substrate-trapping complexes, the better potential functional redundancies between different trimers for dephosphorylation of a given substrate will be avoided, therefore, contributing to a more severe phenotype. This also provides a mechanistic explanation for the apparent lack of correlation between the number of B-type subunit binding deficiencies of a given Aα mutant and the severity of the growth phenotype seen upon its ectopic expression. Rather, the growth phenotypes in our model seem to depend on the number of remaining B' subunit binders, their absolute degree of binding to a given Aα mutant, and the gain-of-binding of Aα mutants to TIPRL1.

Although STRN3 gain-of-binding to mutant Aα could not explain hyperphosphorylation of Akt, p70S6K or GSK3β in mutant Aα expressing HEC-1-A cells, it remains still possible that this gain-of-function mechanism may contribute to the increased oncogenic phenotype of these cells. The role of PP2A-striatins within several identified STRIPAK complexes is indeed considered to be oncogenic, via dephosphorylation of Mst1/2 and Mst3/4 kinases [76], impacting e.g. on regulation of NF-κB (80), Hippo (36,37) and MEK/ERK signaling (75,77,78). Also the recently reported role of PP2A-striatins in regulating cancer cell migration and invasion (81) remains worthwhile to follow-up in future functional assays with mutant Aα expressing HEC-1-A cells, particularly given the known high metastatic potential of serous uterine tumors and carcinosarcomas. The observed reduced phosphorylation of ERK in our HEC-1-A model, found in all mutant Aα expressing cells, would in any case be consistent with a disturbed phosphorylation/dephosphorylation balance within STRIPAK due to enhanced STRN3-PP2A function.

It still remains unclear how changes in the broader (mutant) Aα interactome might additionally contribute to the observed differences in anchorange-independent or tumor growth. Although some of these interaction partners have reported links with cancer pathology, e.g. CCDC6, ANKLE2, TMPO, PP4 and liprin α1/2, this awaits future dedicated functional studies. Likewise, the so far unexplored function of PP2A as an interaction partner of the Integrator complex, a U-rich small nuclear RNAs (UsnRNAs) 3'-end processing factor and RNA polymerase II interacting complex (82), remains worthwhile to further follow-up.

In summary, our data provide biochemical and functional evidence that cancer-associated Aα mutants trigger cancer cell growth of serous uterine carcinoma through a novel dominant negative mechanism dependent on retained binding to tumor suppressive B' subunits, such as B'β, B'γ and B'δ, and gain-of-binding to the TIPRL1 inhibitor. Although the biochemistry of

TIPRL1 remains largely unexplored, it has been hypothesized that TIPRL1 might play a role in PP2A biogenesis as one of the proteins that controls the activity of the newly translated PP2Ac, or alternatively, that it might play a role in PP2Ac inhibition after PP2A holoenzyme disassembly following cellular stresses, such as DNA damage (83). Our current data may support a role of TIPRL1 in the biogenesis of active PP2A trimers through an interaction with Aα. Notably, overexpression of TIPRL1 was reported to result in hyperphosphorylation of p70S6K and 4E-BP (84), consistent with our data in HEC-1-A cells, ectopically expressing Aα mutants with TIPRL1 gain-of-binding. In addition, cBioportal data indicate *TIPRL* overexpression at moderate frequency, and *TIPRL* mutation at low frequency, in several human cancer types (58,59). This all supports a novel pathological role of TIPRL1 in the biology of *PPP2R1A* mutated endometrial cancers, and identifies TIPRL1 as a novel therapeutic anti-cancer target in these hard-to-treat tumors. In this respect, our functional data also clearly highlight the therapeutic potential of treating *PPP2R1A* mutant tumor cells with kinase inhibitors directed against components of the Akt and mTOR/p70S6K signaling pathways, either as monotherapies, or in combination. Our observations predict a good therapeutic efficiency for Akt/mTOR inhibitors and might suggest stratification of patients based on mutant or WT Aα status of the tumor. This opens interesting perspectives for the use of *PPP2R1A* as a therapeutic biomarker, and improved clinical management of these tumors.

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AUTHOR CONTRIBUTIONS

DH, LAA and YH performed experiments for acquisition of biochemical, cellular and *in vivo* data. RD, AH and EW performed and analyzed mass spectrometry experiments. SS and FA provided endometrial cancer cell lines and interpreted data. DH and VJ wrote the manuscript. AS in part supervised the study, designed experiments, interpreted data and revised the manuscript. VJ coordinated the study, in part supervised it, designed experiments and interpreted data.

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SUPPLEMENTARY DATA

<u>Table S1</u>: Overview of *PPP2R1A* mutations found in gynaecological cancers

	Endometrial				Ovarian		
	Endometrioid	**Serous**	**Carcinosarcoma**	**Undifferentiated**	**Endometrioid**	**Serous**	**Clear-cell**
ref. 1							7.1%(3/42) R183G (1) R183W (1) R182W (1)
ref. 2	5% (3/60) R183Q (2) R249H (1)	**40.8%** (20/49) P179R (6) P179L (3) R182W (1) R183W (2) S256F (6) S256Y (1) W257G (1)			12.2% (5/41) P179R (1) R183W (2) S256Y (1) W257G (1)	0% (0/62)	4.1% (2/49) R183W (1) W257C (1) R258C (1)
ref. 3	6.7% (2/30) R183W (1) R182W (1)	**19.2%** (5/26) S256Y (1) P179R (1) S256F (2) W257G (1)			10% (4/40) R183W (2) R183Q (1) R182W (1)	0% (0/91)	9.1% (4/44) R183W (3) R183Q (1)
ref. 4	6.9% (19/276) Low-grade R183Q (4) R183W (4) H87Y (2) R249H (1) R48Q (1) S256F (1) P179L (1) S219L (1) Q237R (1) S219L (1) VE99del (1) 182insV (1) 10% (3/30) High-grade R182W (1) R221W (1) R183W (1)	**43.2%** (16/37) P179R (8) S256F (4) T102K (1) R183W (1) P179L (1) V182M (1)	21.4% (9/42) P179R (4) S256F (2) V229M (1) W257C (1) N312H (1)				

Table S1: continued

	Endometrial				Ovarian		
	Endometrioid	**Serous**	**Carcinosarcoma**	**Undifferentiated**	**Endometrioid**	**Serous**	**Clear-cell**
ref. 5	2.5% (3/118) W257C (1) E216K (1) R258Y (1)	**32%** (8/25) R258H (1) P179R (3) R258Y (1) P179L (2) W257C (1)					
ref. 6		**18.4%** (14/76) P179R (7) R183W (3) S256Y (1) W257G (1) S256F (1) W257C (1)					
ref. 7		**20%** (6/30) P179R (4) S256F (2)					
ref. 8					6.6% (1/15) High-grade P179R (1)	4.5% (1/22) R183W (1)	4.5% (1/22) R183Q (1)
ref. 9					2.7% (1/37) W257C (1)	0% (0/76)	0% (0/43)
ref. 10		**25%** (13/52) P179R (5) S256F (3) W257S (1) W257C (3) L286P (1)					

Table S1: continued

	Endometrial				Ovarian		
	Endometrioid	**Serous**	**Carcinosarcoma**	**Undifferentiated**	**Endometrioid**	**Serous**	**Clear-cell**
ref. 11	4.5% (14/307) S256F (1) R183W (2) R258C (1) R258H (1) R221W (1) L173M (1) A41T (1) A164V (1) R105Q (1) R28C (2) A114V (1) R144C (1) V298L (1) A136T (1) Q237R (1)	**27.9%** (12/43) S256F (2) P179R (7) S256Y (1) L_27splice (1) V220M (1)					
ref. 12,13			26.8% (15/56) P179R (4) S219L (2) R183W (2) Q217K (1) P179L (1) S146L (1) R48Q (1) R183Q (1) S256F (1) S256Y (1) E413K (1) Q288H (1)				
ref. 14				20% (4/20) R183W (1) R182W (1) A252V (1) 22984C>T (1)			

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Table S2: oligonucleotides used for site-directed mutagenesis

Primers Aq mutations	
P179R Forward	5'- GCTCAGATGACACCCGCATGGTGCGGCGGGC -3'
P179R Reverse	5'- GCCCGCCGCACCATGCGGGTGTCATCTGAGC -3'
R182W Forward	5'- ACCCCCATGGTGTGGCGGGCCGCA -3'
R182W Reverse	5'- TGCGGCCCGCCACACCATGGGGGT -3'
R183G Forward	5'- CCCATGGTGCGGGGGGCCGCAGCC -3'
R183G Reverse	5'- GGCTGCGGCCCCCCGCACCATGGG -3'
R183W Forward	5'- ACCCCCATGGTGCGGTGGGCCGCAGCCTCCA -3'
R183W Reverse	5'- TGGAGGCTGCGGCCCACCGCACCATGGGGGT -3'
R183Q Forward	5'- CCCCCATGGTGCGGCAGGCCGCAGCCTCCAA -3'
R183Q Reverse	5'- TTGGAGGCTGCGGCCTGCCGCACCATGGGGG -3'
R249H Forward	5'- TGATGCCCACTCTGCACCAGGCCGCTGAAGA -3'
R249H Reverse	5'- TCTTCAGCGGCCTGGTGCAGAGTGGGCATCA -3'
S256F Forward	5'- CCGCTGAAGACAAGTTCTGGCGCGTCCGCTA -3'
S256F Reverse	5'- TAGCGGACGCGCCAGAACTTGTCTTCAGCGG -3'
S256Y Forward	5'- CCGCTGAAGACAAGTACTGGCGCGTCCGCTA -3'
S256Y Reverse	5'- TAGCGGACGCGCCAGTACTTGTCTTCAGCGG -3'
W257C Forward	5'- TGAAGACAAGTCCTGTCGCGTCCGCTACATG -3'
W257C Reverse	5'- CATGTAGCGGACGCGACAGGACTTGTCTTCA -3'
W257G Forward	5'- GCTGAAGACAAGTCCGGGCGCGTCCGCTACA -3'
W257G Reverse	5'- TGTAGCGGACGCGCCCGGACTTGTCTTCAGC -3'
R258H Forward	5'- AAGACAAGTCCTGGCACGTCCGCTACATGGT -3'
R258H Reverse	5'- ACCATGTAGCGGACGTGCCAGGACTTGTCTT -3'
R418W Forward	5'- GACGCCAAGTGGTGGGTGCGGCTGG -3'
R418W Reverse	5'- CCAGCCGCACCCACCACTTGGCGTC -3'
L/A410stop forward	5'- GTTCCAGATTACGCTTCTAGAATGGCGGCGGCCGA CGGCGACG -3'
L/A410stop reverse	5'- TATCTTATCATGTCTGGATCCTCACAGCTCCACAATG GCAGGGAGC -3'

S3: Excel spreadsheet, with raw and processed data from MS Orbitrap experiments

	Normalized abundance						Ratios mutant as to WT						Ratios normalized for *PPP2R1A* input					
	WTAalfa	P179R	R182W	R183Q	R183G	S256F	WTAalfa	P179R	R182W	R183Q	R183G	S256F	WTAalfa	P179R	R182W	R183Q	R183G	S256F
SSR4	2,94E+05	2,61E+05	1,23E+06	2,48E+05	1,76E+05	3,99E+05	1,0	0,9	4,2	0,8	0,6	1,4	1,00	1,76	2,20	0,21	0,54	0,62
CNIH4	1,85E+05	1,00E+05	9,83E+05	1,23E+05	1,07E+05	2,63E+05	1,0	0,5	5,3	0,7	0,6	1,4	1,00	1,08	2,80	0,16	0,52	0,65
INT9	5,04E+05	2,43E+05	1,54E+05	4,74E+05	5,72E+05	3,49E+05	1,0	0,5	0,3	0,9	1,1	0,7	1,00	0,96	0,16	0,23	1,03	0,32
STK24	2,55E+05	2,24E+05	2,88E+05	2,82E+05	3,04E+05	2,74E+05	1,0	0,9	1,1	1,1	1,2	1,1	1,00	1,75	0,59	0,27	1,08	0,49
CNN2	1,75E+05	2,65E+05	3,55E+05	1,38E+05	1,36E+05	4,00E+05	1,0	1,5	2,0	0,8	0,8	2,3	1,00	3,00	1,06	0,19	0,70	1,04
TFRC	2,81E+05	2,05E+05	1,91E+05	6,26E+04	1,20E+05	2,26E+05	1,0	0,7	0,7	0,2	0,4	0,8	1,00	1,45	0,36	0,05	0,38	0,37
PPIA	3,01E+05	7,67E+04	1,06E+05	8,66E+04	8,74E+04	3,22E+05	1,0	0,3	0,4	0,3	0,3	1,1	1,00	0,51	0,19	0,07	0,26	0,49
S100A11	4,31E+05	9,77E+05	4,65E+05	2,14E+05	2,63E+05	5,93E+05	1,0	2,3	1,1	0,5	0,6	1,4	1,00	4,52	0,57	0,12	0,55	0,63
MYO1C	1,82E+05	2,13E+05	1,66E+06	3,51E+05	3,28E+05	8,24E+05	1,0	1,2	9,1	1,9	1,8	4,5	1,00	2,33	4,79	0,47	1,62	2,06
OMA1	1,92E+04	3,42E+04	1,12E+06	5,29E+04	2,89E+04	4,52E+04	1,0	1,8	58,3	2,8	1,5	2,4	1,00	3,55	30,70	0,68	1,36	1,07
TIPRL	5,84E+04	7,34E+04	2,76E+05	1,63E+06	2,44E+05	2,08E+05	1,0	1,3	4,7	27,9	4,2	3,6	1,00	2,51	2,49	6,86	3,77	1,62
SLC27A2	1,44E+05	2,11E+05	8,13E+05	1,62E+05	1,25E+05	1,91E+05	1,0	1,5	5,6	1,1	0,9	1,3	1,00	2,91	2,96	0,28	0,78	0,60
PREB	7,52E+04	3,34E+03	4,13E+03	2,52E+05	3,35E+04	6,61E+03	1,0	0,0	0,1	3,4	0,4	0,1	1,00	0,09	0,03	0,82	0,40	0,04
DDOST	7,97E+04	4,86E+04	1,79E+05	3,30E+04	1,58E+04	1,37E+05	1,0	0,6	2,3	0,4	0,2	1,7	1,00	1,21	1,18	0,10	0,18	0,78
POR	2,10E+04	1,27E+04	2,58E+04	1,86E+04	1,10E+03	4,44E+04	1,0	0,6	1,2	0,9	0,1	2,1	1,00	1,21	0,65	0,22	0,05	0,97
OSBPL8	3,68E+04	1,38E+04	6,14E+04	1,07E+05	3,18E+04	6,52E+03	1,0	0,4	1,7	2,9	0,9	0,2	1,00	0,75	0,88	0,72	0,78	0,08
HSD17B10	8,34E+04	8,40E+04	5,12E+04	4,24E+04	1,65E+04	8,99E+04	1,0	1,0	0,6	0,5	0,2	1,1	1,00	2,00	0,32	0,12	0,18	0,49
TMEM205	8,99E+04	5,76E+04	8,55E+04	3,87E+04	1,82E+04	1,47E+05	1,0	0,6	1,0	0,4	0,2	1,6	1,00	1,28	0,50	0,11	0,18	0,74
LACTB	3,60E+05	1,04E+05	7,75E+04	4,98E+04	5,20E+04	1,30E+05	1,0	0,3	0,2	0,1	0,1	0,4	1,00	0,58	0,11	0,03	0,13	0,17
TMX3	7,11E+04	6,56E+04	3,66E+05	0,00E+00	1,45E+04	8,04E+04	1,0	0,9	5,1	0,0	0,2	1,1	1,00	1,84	2,71	0,00	0,18	0,52
RAB10	1,56E+03	2,75E+03	0,00E+00	0,00E+00	1,26E+03	1,07E+05	1,0	1,8	0,0	0,0	0,8	68,3	1,00	3,51	0,00	0,00	0,73	31,17
HADHB	2,12E+04	2,94E+04	2,60E+04	9,77E+03	6,60E+03	5,27E+04	1,0	1,4	1,2	0,5	0,3	2,5	1,00	2,75	0,64	0,11	0,28	1,13
PPP2R5B	9,67E+04	1,00E-01	1,96E+03	0,00E+00	7,02E+01	1,35E+04	1,0	0,0	0,0	0,0	0,0	0,1	1,00	0,00	0,01	0,00	0,00	0,06
DHX15	1,00E+00	4,48E+02	6,33E+02	0,00E+00	0,00E+00	5,46E+04	1,0	447,7	632,7	0,0	0,0	54561,3	1,00	891,24	332,90	0,00	0,00	24899,10
KPNA4	8,27E+04	1,15E+04	3,58E+04	1,31E+04	4,90E+03	2,45E+04	1,0	0,1	0,4	0,2	0,1	0,3	1,00	0,28	0,23	0,04	0,05	0,14
SLC3A2	4,00E+03	1,87E+03	1,12E+05	2,43E+03	2,93E+03	1,12E+04	1,0	0,5	28,1	0,6	0,7	2,8	1,00	0,93	14,79	0,15	0,66	1,28
DHRS7B	2,49E+04	1,38E+04	1,28E+05	5,80E+03	3,37E+03	2,31E+04	1,0	0,6	5,1	0,2	0,1	0,9	1,00	1,11	2,69	0,06	0,12	0,42
ATP5O	1,45E+04	3,64E+04	3,62E+04	0,00E+00	3,46E+03	0,00E+00	1,0	2,5	2,5	0,0	0,2	0,0	1,00	4,98	1,31	0,00	0,21	0,00
RHOF	3,00E+03	4,37E+03	1,60E+05	0,00E+00	5,98E+03	5,11E+03	1,0	1,5	53,3	0,0	2,0	1,7	1,00	2,90	28,03	0,00	1,80	0,78
CAPN1	9,51E+03	3,67E+02	5,56E+02	2,04E+02	2,31E+02	8,26E+04	1,0	0,0	0,1	0,0	0,0	8,7	1,00	0,08	0,03	0,01	0,02	3,96
PIGS	2,90E+03	6,84E+03	8,04E+04	1,85E+03	2,74E+02	0,00E+00	1,0	2,4	27,8	0,6	0,1	0,0	1,00	4,70	14,61	0,16	0,09	0,00
ALDH18A1	3,99E+04	2,43E+04	2,72E+04	1,24E+04	8,34E+03	5,56E+04	1,0	0,6	0,7	0,3	0,2	1,4	1,00	1,21	0,36	0,08	0,19	0,64
PPFIBP1	1,17E+05	8,61E+03	6,40E+04	0,00E+00	4,72E+03	5,95E+04	1,0	0,1	0,5	0,0	0,0	0,5	1,00	0,15	0,29	0,00	0,04	0,23
PA2G4	6,36E+04	5,49E+04	5,79E+04	2,80E+04	1,75E+04	1,34E+05	1,0	0,9	0,9	0,4	0,3	2,1	1,00	1,72	0,48	0,11	0,25	0,96
OPA1	2,51E+04	3,14E+03	2,13E+04	3,24E+03	1,37E+04	4,42E+04	1,0	0,1	0,8	0,1	0,5	1,8	1,00	0,25	0,45	0,03	0,49	0,80
SHMT2	9,95E+04	1,19E+05	9,00E+04	4,06E+04	3,94E+04	1,52E+05	1,0	1,2	0,9	0,4	0,4	1,5	1,00	2,39	0,48	0,10	0,36	0,70
S100A6	3,10E+05	1,02E+05	1,54E+05	7,30E+04	7,67E+04	3,08E+05	1,0	0,3	0,5	0,2	0,2	1,0	1,00	0,66	0,26	0,06	0,22	0,45

	Normalized abundance						Ratios mutant as to WT						Ratios normalized for *PPP2R1A* input					
	WTAalfa	P179R	R182W	R183Q	R183G	S256F	WTAalfa	P179R	R182W	R183Q	R183G	S256F	WTAalfa	P179R	R182W	R183Q	R183G	S256F
PDCD10	1,78E+05	1,17E+05	1,22E+05	1,08E+05	1,44E+05	1,15E+05	1,0	0,7	0,7	0,6	0,8	0,7	1,00	1,31	0,36	0,15	0,73	0,30
GRSF1	8,80E+04	2,10E+05	1,06E+05	4,30E+04	7,86E+04	8,24E+04	1,0	2,4	1,2	0,5	0,9	0,9	1,00	4,76	0,63	0,12	0,81	0,43
AIML1L	5,32E+05	3,38E+04	3,97E+04	5,80E+04	2,62E+04	1,81E+05	1,0	0,1	0,1	0,1	0,0	0,3	1,00	0,13	0,04	0,03	0,04	0,16
HSD17B11	9,94E+04	5,79E+04	3,32E+05	6,56E+04	5,23E+04	1,35E+05	1,0	0,6	3,3	0,7	0,5	1,4	1,00	1,16	1,76	0,16	0,47	0,62
BSG	4,62E+04	5,14E+04	1,86E+05	2,89E+05	4,41E+04	2,40E+05	1,0	1,1	4,0	6,3	1,0	5,2	1,00	2,21	2,11	1,54	0,86	2,37
RHBDD2	2,28E+03	1,82E+04	5,18E+05	2,79E+03	1,56E+04	0,00E+00	1,0	8,0	227,4	1,2	6,8	0,0	1,00	15,94	119,63	0,30	6,17	0,00
SSR1	9,97E+04	1,12E+05	6,49E+05	8,89E+04	1,91E+04	6,39E+04	1,0	1,1	6,5	0,9	0,2	0,6	1,00	2,24	3,42	0,22	0,17	0,29
QPCTL	1,75E+04	4,60E+04	2,84E+05	4,24E+04	4,02E+04	4,51E+04	1,0	2,6	16,2	2,4	2,3	2,6	1,00	5,23	8,53	0,59	2,07	1,18
INTS3	1,27E+05	1,99E+05	1,57E+04	2,37E+04	2,20E+05	3,86E+04	1,0	1,6	0,1	0,2	1,7	0,3	1,00	3,11	0,06	0,05	1,56	0,14
FGFR1OP	2,84E+05	5,07E+04	6,33E+04	1,78E+05	9,93E+04	2,56E+05	1,0	0,2	0,2	0,6	0,4	0,9	1,00	0,36	0,12	0,15	0,32	0,41
INTS4	1,40E+06	1,00E+06	5,52E+05	1,41E+06	1,99E+06	8,24E+05	1,0	0,7	0,4	1,0	1,4	0,6	1,00	1,42	0,21	0,25	1,28	0,27
PPP2R5E	1,03E+07	2,54E+05	7,67E+05	2,07E+05	2,02E+05	3,13E+06	1,0	0,0	0,1	0,0	0,0	0,3	1,00	0,05	0,04	0,00	0,02	0,14
SLC25A5	8,03E+05	6,94E+05	2,89E+06	5,86E+05	4,78E+05	9,41E+05	1,0	0,9	3,6	0,7	0,6	1,2	1,00	1,72	1,89	0,18	0,54	0,53
INTS6	1,23E+06	7,76E+05	3,12E+05	1,80E+06	2,04E+06	7,46E+05	1,0	0,6	0,3	1,5	1,7	0,6	1,00	1,26	0,13	0,36	1,49	0,28
PPP2R2D	1,25E+06	1,27E+04	3,61E+04	5,36E+04	1,39E+04	6,53E+04	1,0	0,0	0,0	0,0	0,0	0,1	1,00	0,02	0,02	0,01	0,01	0,02
PPP2R5A	5,27E+06	8,00E+04	1,31E+05	1,22E+05	8,38E+04	5,35E+05	1,0	0,0	0,0	0,0	0,0	0,1	1,00	0,03	0,01	0,01	0,01	0,05
RPN2	1,80E+06	1,72E+06	3,53E+06	7,32E+05	6,52E+05	2,45E+06	1,0	1,0	2,0	0,4	0,4	1,4	1,00	1,90	1,03	0,10	0,33	0,62
IDH3B	4,88E+05	5,83E+05	9,85E+06	4,68E+05	2,04E+06	9,41E+05	1,0	1,2	20,2	1,0	4,2	1,9	1,00	2,38	10,61	0,24	3,77	0,88
STRN3	4,55E+06	3,36E+06	9,52E+06	9,80E+06	8,36E+06	8,02E+06	1,0	0,7	2,1	2,2	1,8	1,8	1,00	1,47	1,10	0,53	1,66	0,80
ATP1A1	6,57E+05	4,86E+05	2,43E+06	5,08E+05	3,36E+05	9,07E+05	1,0	0,7	3,7	0,8	0,5	1,4	1,00	1,47	1,94	0,19	0,46	0,63
SLC25A6	2,24E+05	2,59E+05	1,56E+06	1,58E+05	1,64E+05	2,58E+05	1,0	1,2	7,0	0,7	0,7	1,2	1,00	2,30	3,67	0,17	0,66	0,53
ALDH3A2	1,84E+05	2,00E+05	1,11E+07	2,32E+05	1,66E+05	1,23E+06	1,0	1,1	60,5	1,3	0,9	6,7	1,00	2,16	31,81	0,31	0,81	3,05
SLC25A3	3,86E+05	3,09E+05	1,94E+06	3,80E+05	2,14E+05	3,16E+05	1,0	0,8	5,0	1,0	0,6	0,8	1,00	1,59	2,65	0,24	0,50	0,37
PPFIA1/2	7,05E+06	5,01E+05	4,51E+06	4,10E+06	1,15E+06	6,15E+06	1,0	0,1	0,6	0,6	0,2	0,9	1,00	0,14	0,34	0,14	0,15	0,40
MYH9	8,22E+06	7,80E+06	3,43E+06	1,07E+06	6,41E+06	2,29E+06	1,0	0,9	0,4	0,1	0,8	0,3	1,00	1,89	0,22	0,03	0,70	0,13
PPP2R5D	4,46E+07	7,87E+06	2,30E+07	4,70E+07	1,39E+07	2,49E+07	1,0	0,2	0,5	1,1	0,3	0,6	1,00	0,35	0,27	0,26	0,28	0,26
PPP4R1	2,55E+06	3,01E+06	6,78E+05	1,50E+06	1,18E+06	2,48E+07	1,0	1,2	0,3	0,6	0,5	9,7	1,00	2,36	0,14	0,14	0,42	4,45
PPP2R2A/B	3,47E+07	3,37E+05	2,94E+05	3,21E+05	3,08E+05	5,30E+05	1,0	0,0	0,0	0,0	0,0	0,0	1,00	0,02	0,00	0,00	0,01	0,01
PPP2R1A	3,21E+09	1,60E+09	6,06E+09	1,33E+10	3,53E+09	7,10E+09	1,0	0,5	1,9	4,1	1,1	2,2	1,00	0,99	0,99	1,02	0,99	1,01
STRN	1,70E+07	1,27E+07	2,87E+07	3,25E+07	2,89E+07	2,82E+07	1,0	0,7	1,7	1,9	1,7	1,7	1,00	1,48	0,89	0,47	1,53	0,75
CCDC6	1,80E+07	2,13E+07	7,16E+06	1,67E+07	1,16E+07	1,20E+08	1,0	1,2	0,4	0,9	0,6	6,6	1,00	2,36	0,21	0,23	0,58	3,03
STRIP1/2	3,73E+06	4,01E+06	8,67E+06	7,62E+06	7,37E+06	5,75E+06	1,0	1,1	2,3	2,0	2,0	1,5	1,00	2,14	1,22	0,50	1,78	0,70
SLMAP	7,58E+06	6,83E+06	1,51E+07	1,37E+07	1,40E+07	1,22E+07	1,0	0,9	2,0	1,8	1,8	1,6	1,00	1,79	1,05	0,44	1,67	0,73
ANKLE2	1,97E+06	1,44E+06	1,20E+06	1,08E+06	2,27E+07	1,44E+06	1,0	0,7	0,6	0,6	11,5	0,7	1,00	1,45	0,32	0,14	10,42	0,33
STRN4	6,12E+06	4,85E+06	1,19E+07	1,25E+07	1,00E+07	9,37E+06	1,0	0,8	1,9	2,0	1,6	1,5	1,00	1,58	1,02	0,50	1,48	0,70
FECH	1,58E+07	5,54E+05	3,09E+06	4,92E+07	2,87E+07	3,37E+07	1,0	0,0	0,2	3,1	1,8	2,1	1,00	0,07	0,10	0,76	1,64	0,98
PPP2CA	1,40E+05	8,86E+03	5,69E+04	7,82E+05	2,82E+04	2,05E+06	1,0	0,1	0,4	5,6	0,2	14,6	1,00	0,13	0,21	1,37	0,18	6,68
INTS1	2,24E+06	1,66E+06	7,96E+05	2,52E+06	3,80E+06	1,20E+06	1,0	0,7	0,4	1,1	1,7	0,5	1,00	1,47	0,19	0,28	1,53	0,24

	Normalized abundance						Ratios mutant as to WT						Ratios normalized for *PPP2R1A* input					
	WTAalfa	P179R	R182W	R183Q	R183G	S256F	WTAalfa	P179R	R182W	R183Q	R183G	S256F	WTAalfa	P179R	R182W	R183Q	R183G	S256F
PPP2CB	3,49E+06	1,56E+06	1,46E+06	4,10E+06	2,12E+06	4,79E+06	1,0	0,4	0,4	1,2	0,6	1,4	1,00	0,89	0,22	0,29	0,55	0,63
PPP2R5C	1,06E+07	2,96E+05	1,91E+06	2,81E+06	8,71E+05	2,60E+06	1,0	0,0	0,2	0,3	0,1	0,2	1,00	0,06	0,09	0,06	0,07	0,11
PPP4C	3,43E+05	1,91E+05	1,78E+04	2,50E+05	6,95E+04	2,70E+06	1,0	0,6	0,1	0,7	0,2	7,9	1,00	1,11	0,03	0,18	0,18	3,59
TMEM43	4,67E+05	3,06E+05	1,92E+06	5,37E+05	6,15E+05	8,52E+05	1,0	0,7	4,1	1,2	1,3	1,8	1,00	1,30	2,17	0,28	1,19	0,83
MOB4	3,19E+06	2,36E+06	6,32E+06	8,57E+06	6,50E+06	4,69E+06	1,0	0,7	2,0	2,7	2,0	1,5	1,00	1,48	1,04	0,66	1,84	0,67
FKBP8	3,25E+05	1,94E+05	5,45E+05	1,63E+05	1,29E+05	1,43E+05	1,0	0,6	1,7	0,5	0,4	0,4	1,00	1,19	0,88	0,12	0,36	0,20
CTTNBP2NL	4,67E+05	2,20E+05	6,13E+05	5,62E+05	5,03E+05	5,01E+05	1,0	0,5	1,3	1,2	1,1	1,1	1,00	0,94	0,69	0,30	0,97	0,49
CCT5	4,68E+05	5,43E+05	9,34E+05	3,53E+05	3,17E+05	6,91E+05	1,0	1,2	2,0	0,8	0,7	1,5	1,00	2,31	1,05	0,18	0,61	0,67
TMPO	5,25E+05	7,04E+05	8,72E+05	3,70E+05	5,35E+05	4,11E+05	1,0	1,3	1,7	0,7	1,0	0,8	1,00	2,67	0,87	0,17	0,92	0,36
QSOX2	3,98E+05	2,23E+05	1,56E+06	7,11E+05	1,89E+05	6,47E+05	1,0	0,6	3,9	1,8	0,5	1,6	1,00	1,12	2,06	0,44	0,43	0,74
NCEH1	2,54E+05	2,99E+05	4,85E+05	2,63E+05	1,76E+05	3,65E+05	1,0	1,2	1,9	1,0	0,7	1,4	1,00	2,35	1,01	0,25	0,63	0,66
ARF4	1,78E+05	1,35E+05	4,01E+05	9,54E+04	9,95E+04	1,72E+05	1,0	0,8	2,2	0,5	0,6	1,0	1,00	1,50	1,18	0,13	0,50	0,44
FGFR1OP2	1,82E+06	1,42E+06	3,52E+06	3,66E+06	3,28E+06	2,38E+06	1,0	0,8	1,9	2,0	1,8	1,3	1,00	1,56	1,02	0,49	1,63	0,60
INTS7	7,08E+05	5,89E+05	2,60E+05	6,02E+05	1,01E+06	3,75E+05	1,0	0,8	0,4	0,9	1,4	0,5	1,00	1,66	0,19	0,21	1,28	0,24
INTS5	3,42E+05	3,75E+05	8,96E+04	3,95E+05	8,27E+05	2,03E+05	1,0	1,1	0,3	1,2	2,4	0,6	1,00	2,18	0,14	0,28	2,18	0,27
INTS8	8,78E+05	6,72E+05	1,91E+05	9,62E+05	1,28E+06	4,43E+05	1,0	0,8	0,2	1,1	1,5	0,5	1,00	1,52	0,11	0,27	1,32	0,23
INTS2	7,55E+05	5,78E+05	5,05E+05	7,42E+05	9,05E+05	6,13E+05	1,0	0,8	0,7	1,0	1,2	0,8	1,00	1,52	0,35	0,24	1,08	0,37
ATP2A2/1	7,22E+05	6,03E+05	2,66E+06	5,22E+05	4,32E+05	8,83E+05	1,0	0,8	3,7	0,7	0,6	1,2	1,00	1,66	1,94	0,18	0,54	0,56
CPSF3L	8,16E+05	6,10E+05	4,28E+05	7,48E+05	1,25E+06	4,08E+05	1,0	0,7	0,5	0,9	1,5	0,5	1,00	1,49	0,28	0,23	1,38	0,23
ASPH	2,37E+06	1,91E+06	4,05E+06	1,99E+06	1,20E+06	3,24E+06	1,0	0,8	1,7	0,8	0,5	1,4	1,00	1,60	0,90	0,21	0,46	0,62
SIKE1	1,17E+06	7,24E+05	1,61E+06	1,75E+06	1,41E+06	2,36E+06	1,0	0,6	1,4	1,5	1,2	2,0	1,00	1,24	0,73	0,37	1,09	0,92

7 General conclusions and discussion

GENERAL CONCLUSIONS AND DISCUSSION

PP2A represents one of the major protein phosphatase families in the cell. Each of the about 100 different PP2A heterotrimeric phosphatases is comprised of a catalytic subunit bridged by the A scaffold subunit to a specific regulatory B subunit, the later determining the physiological function, the tissue distribution and the subcellular localization of the holoenzyme. Alterations in PP2A function and/or activity have been reported in many human pathologies [9-12]. **In this thesis, we report the biochemical and functional characterization of PP2A aberrations found in patients suffering from ID and endometrial cancer.** The co-occurrence of identical Aα mutations in both pathologies suggested a common underlying disease mechanism. Indeed, we provide evidence for a common dominant-negative mode-of-action of these mutations.

PP2A dysfunction, a new causative mechanism for ID

Recently, large whole exome (and genome) sequencing projects have revealed *de novo* mutations in coding regions as an important cause of severe ID. In collaboration with a large European consortium of clinical geneticists, we identified and biochemically characterized mutations in *PPP2R5D* (P53S, E198K, E200K, P201R and W207R), encoding the regulatory B′δ subunit, and in *PPP2R1A* (P179L, R182W and R258H), encoding the Aα subunit, in 16 individuals with a distinct form of ID. The main clinical features were mild to severe ID, long-lasting hypotonia, susceptibility for epilepsy, frontal bossing, mild hypertelorism and downslanting palpebral fissures, and for the *PPP2R5D* cases (except P53S), macrocephaly. The mutations, identified via parent-child trio exome sequencing, appear clearly clustered and some are recurrent. Moreover, for several patients, the PP2A mutation was the only unique aberration identified in the affected child, all highly suggestive for causality [165]. For all but one (P53S) of the discovered *de novo* mutations, our biochemical data are consistent with a common dominant-negative disease-causing mechanism, affecting PP2A B′δ-dependent dephosphorylation. All *PPP2R5D* mutants (except P53S) indeed no longer bound PP2A A and C subunits, while all *PPP2R1A* mutants remarkably retained binding to B′δ, but formed catalytically impaired complexes with this subunit. Therefore, the hypothesis emerged that A-C binding defective B′δ mutants (except P53S), or catalytically impaired mutant Aα-B′δ complexes, may indeed block dephosphorylation of B′δ-specific substrates and interfere with phosphorylation-dephosphorylation dynamics in the brain by a competition mechanism with the WT B′δ and WT Aα containing PP2A trimers.

The B′δ mutant P53S is atypical and this case also had a different clinical picture including a short stature and microcephaly. The P53 residue localizes in the B′δ-specific N-terminal domain, a region likely involved in substrate binding. So in theory, P53S might change the interaction of B′δ with relevant substrates or might introduce a new phosphorylation site that affects regulation by protein kinases. Such changes could also easily have a gain-of-function or dominant-negative effect. All other ID-associated B′δ mutant residues (E198K, E200K, P201R and W207R) cluster in an acidic loop directly facing the A and C subunits. The E198 residue makes direct contact with the C subunit and the mutation of this residue, the most recurrent *PPP2R5D* mutation identified, shows also the most severe biochemical defects and

the most severe phenotype in the patients. Therefore, a clear correlation exists between the ID degree and the biochemical disturbance of PP2A. Remarkably, mutations have been reported in this conserved loop in other regulatory B subunits, B'β and B'γ (S161L in *PPP2R5B* and T157del in *PPP2R5C*), in overgrowth patients [152]. This again underscores the importance of this conserved loop in PP2A holoenzyme formation and likely expands the involvement of PP2A in ID and overgrowth pathogenesis to multiple B' regulatory subunits, although, until now, the B'β and B'γ mutations have only been found in single cases. The future biochemical and functional characterization of both overgrowth-associated B'β and B'γ mutants should provide further insight in this.

Potential PP2A substrates affected in ID

PPP2R5D encodes the longest isoform of the B56/PR61/B' family of PP2A regulatory subunits and is highly expressed in brain, particularly in the striatum [37]. B'δ also localizes both to the nucleus and the cytoplasm. Some physiological roles of B'δ in the brain are well documented:

(1) In concordance with a previous report [59, 166], we show that overexpression of mutant B'δ or mutant Aα causes hyperphoshorylation, and thus inactivation, of **GSK3β** in HEK293 cells, suggesting that PP2A-B'δ is a GSK3β Ser9 phosphatase in these cells. While PI3K-Akt3 inactivates GSK3β via Ser9 phosphorylation, PP2A-B'δ may thus be the counteracting GSK3β phosphatase in brain. Cyclin D2 (*CCND2*), which can stimulate neuronal progenitor cells to progress through the cell cycle, is an important neuronal substrate of GSK3β, with its phosphorylation leading to degradation [167]. Interestingly, *de novo* mutations at different levels of the PI3K-Akt3-GSK3β-cyclin D2 signaling pathway cause megalencephaly syndromes (Figure 6). Germline mutations of *PIK3R2* and *AKT3* result in megalencephaly-capillary malformation syndrome (MCAP), postzygotic mutations of *PIK3CA* and germline mutations in *CCND2* cause megalencephaly-polymicrogyria-polydactyly-hydrocephalus syndrome (MPPH), and postzygotic mutations of *PIK3CA* and *AKT3* cause isolated hemimegalencephaly. All these aberrations eventually cause accumulation of Cyclin D2 leading to brain overgrowth (Figure 6) [167]. Phenotypically, MCAP/MPPH patients share several characteristics with the mutant B'δ and Aα cases, for example ID, hypotonia, tendency to develop epilepsy, and several dysmorphic facial features like tented upper lip and deep-set eyes, suggesting that in B56deltopathy patients, inactivation of GSK3β may also result in increased cyclin D2 expression. In contrast, our patients lack polymycrogyria and vascular abnormalities [168]. All together, this suggests an important involvement of defective B'δ-dependent GSK3β control in B56deltopathy patients, but also highlights the possible involvement of other dysfunctional PP2A-B'δ substrates given the similar, but non-identical features compared to MCAP/MPPH.

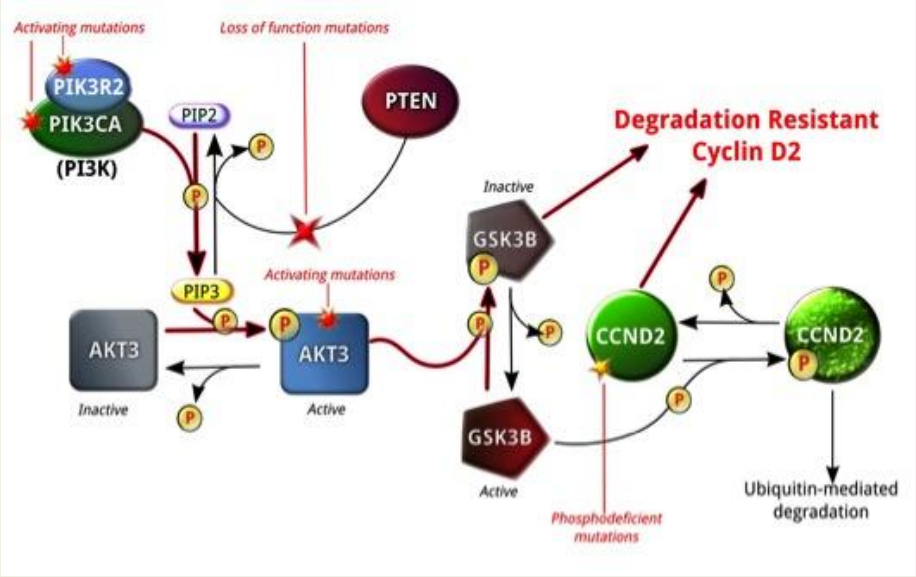

Figure 6: Overview of the PI3K-Akt3-GSK3β-cyclin D2 signaling pathway, indicating the mutated proteins leading to megalencephaly syndromes. Figure adapted from [167].

(2) B'δ stimulates dopaminergic signaling by dephosphorylating dopamine & cAMP regulated phosphoprotein of 32kDa (**DARPP32**), both in the nucleus and in the cytoplasm. DARPP32 plays a critical role in the functions of dopamine (motor behavior and reward-driven learning), but also of antipsychotic drugs and drugs of abuse. In the cytoplasm, stimulation of dopamine 1 receptors increases the level of cAMP resulting in PKA activation and DARPP32 phosphorylation at Thr34 [169]. This activating phoshorylation stimulates DARPP32 to inhibit PP1 resulting in increased phosphorylation of substrates like ion pumps, protein kinases, voltage-gated ion channels, etc. Cdk5-mediated phosphorylation of Thr75 blocks the Thr34 phosphorylation by PKA under basal conditions. After D1R stimulation, PKA phosphorylates B'δ at Ser566 which stimulates B'δ-mediated dephosphorylation of DARPP32 Thr75. In the nucleus, B'δ Ser566 phoshorylation by cAMP/PKA results in dephosphorylation of DARPP32 Ser97 and its nuclear accumulation [170]. In addition, PP2A-PR72 mediates Ca^{2+}-dependent dephosphorylation of DARPP-32 Thr-75 upon activation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) or N-methyl-D-aspartate receptors (NMDA) receptors, both glutamate receptors and non-selective cation channels [171]. Thus, PP2A-B'δ plays a major role in dopaminergic signaling and control of DARPP32 function in the striatum, which may be affected in B56deltopathy patients.

(3) Studies in B'δ knockout mice revealed that the microtubule-associated tau protein becomes hyperphosphorylated at pathological residues in restricted brain areas (mainly brain stem and upper spinal cord), accompanied by pretangle conformation but without neurofibrillary tangle formation [59]. PP2A-B'δ is a poor tau phosphatase *in vitro*, arguing against a direct effect. Rather, the cellular activator of CDK5, p35, was strikingly absent in the affected brain areas, resulting in decreased activity of CDK5. In contrast to tau, **p35** is an excellent *in vitro* PP2A-B'δ substrate. Behavioral tests indicated impaired sensorimotor but normal cognitive functions, which is in contrast with the severe ID seen in most B56deltopathy patients. The appearance of very discrete neurological phenotypes in the KO

mouse (in contrast to the widespread distribution of B'δ in WT brain) indicates functional redundancy of other PP2A complexes. Accordingly, KI of the ID-associated B'δ and Aα mutations might indeed be more severe than complete B'δ KO, because in these cases, the substrate-trapping mutant proteins may actively protect/shield substrates from redundant phosphatases.

(4) B'δ controls **Tyrosine Hydroxylase** (TH), the rate-limiting enzyme for dopamine (and other catecholamines), both at the protein and gene expression levels. It was shown that PKCδ-mediated phoshorylation of B'δ Ser566 stimulates dephosphorylation of the Ser40 residue of TH, resulting in its inhibition [172]. PP2A redundancy occurs toward TH, since the B'β isoform is also able to dephosphorylate TH Ser40 [173]. Furthermore, nuclear translocation of B'δ is triggered by activation of voltage-gated calcium signals of which a subunit, β4, can bind B'δ and brings it into the nucleus. Together with accessory proteins, B'δ binds the TH promotor, dephosphorylates Histon H3 and represses gene expression of TH [174]. It remains to be determined whether TH function and/or expression are affected in B56deltopathy patients.

(5) The **Nerve Growth Factor** (NGF) signaling pathway controls neuronal development, survival and plasticity of most neuronal populations [175]. Within this pathway, B'δ- and B'β-containing PP2A complexes are able to dephosphorylate the NGF receptor, tropomyosin-related kinase A (TrkA), at Ser and Thr residues, potentiating the intrinsic Tyr kinase activity of TrkA. This enhances NGF signaling via Akt and Ras pathways to promote neuritogenesis and neuronal differentiation. B'δ dysfunction may therefore impair NGF signaling.

(6) Phosphorylation/dephosphorylation events regulate the dimerisation and function of **HAND 1 and HAND 2** transcription factors, which are required for neuronal, limb, vascular, heart and extraembryonic development [176]. While HAND phosphorylation is mediated by PKA and PKC, PP2A-B'δ is responsible for their dephosphorylation. B'δ is abundant in undifferentiated embryonic cells and is downregulated during differentiation. Moreover, overexpression of HAND factors in de developing limbs of chick and mice causes polydactyly, a condition in which more than 10 finger or toes are developed. This condition was also found in one Aα case. Nevertheless, overall brain development seems largely unaffected in the majority of B56deltopathy cases.

(7) Finally, B'δ also constitutes part of the neural variant of the muscle cAMP-dependent PKA anchoring protein (**mAKAP**) complex. The mAKAP scaffold protein also binds several other phosphatases (PP1, PP2B) and kinases (PKA, PDK1, RSK3, ERK5), integrating local signaling events. In response to elevated levels of cAMP, PKA is able to phosphorylate the alternatively spliced type 4 phosphodiesterase isoform D3 (PDE4D3), stimulating its activity. On the other hand, PKA also phosphorylates B'δ, enhancing its dephoshorylation activity towards PDE4D3, and subsequently inhibiting it [177]. mAKAPα and mAKAPβ are two alternatively spliced isoforms of mAKAP expressed in the heart and the brain, respectively. The presence of B'δ in mAKAPβ, once again, suggests an important regulatory role of this PP2A subunit in dopaminergic signaling.

The identification of the physiological PP2A-B′δ substrates, whose phosphorylation-dephosphorylation dynamics may be deregulated in B56deltopathy patients, clearly awaits further biochemical analyses (for instance, through identification of B′δ interacting proteins in neurons), and definitely, establishment of appropriate cellular and *in vivo* models of the disease (cfr. Future perspectives), which can be further studied in great detail.

Therapeutic options for B56deltopathy patients?

Given the known redundancy of B subunits for several (neuronal) substrates, it is still unclear which substrates are primarily affected by B′δ and Aα mutations and, hence, are crucial to the pathology. This hampers the development of treatment protocols to alleviate symptoms and provide ID patients with a better quality of life. Nevertheless, since overall brain development of the B56deltopathy patients seems largely unaffected, there is perhaps hope to restore or improve dysfunctional phosphorylation-dephosphorylation dynamics through administration of specific pharmaca. While use of kinase inhibitors has clearly a proven clinical benefit in certain diseases (particularly cancer), more and more phosphatase modulators with a therapeutic potential are currently being discovered [178]. Recent developments in the PP2A field have led to the discovery of PP2A activating drugs ('PADs'), such as FTY720, phenothiazines and their derivates [116, 162]. It would be of enormous therapeutic interest to test whether these compounds, all already FDA-approved, might improve brain functions or restore neuronal signaling in the B56deltopathy patients. In addition, we speculate that specific kinase inhibitors (potentially in combination with a PAD) may also be excellent candidate-drugs to normalise phosphorylation of dysregulated PP2A-B56δ substrates. This however awaits identification of the kinases whose function is counteracted by PP2A-B′δ in brain.

***PPP2R1A* mutations function via a dominant-negative mode-of-action in uterine cancer**

The co-occurrence of identical Aα mutations in both ID and serous endometrial cancer suggested a common underlying dominant-negative disease mechanism, which we further and more extensively investigated in the cancer context.

Our cellular binding assays in HEK293 and HEC-1-A cells, ectopically expressing Aα mutant and WT proteins, reveal some very interesting observations, shaking up the traditional way we think about PP2A complex formation.
(1) **Isoform-specific differences** in binding behavior to cancer-derived Aα mutants exist within members of the same B-type subunit family. This is especially true for the B56/PR61/B' family since B′δ seems to behave differently than the other members; in fact, B′δ is the only B-type subunit that still significantly binds all mutant proteins, while the other family members show a more heterogeneous binding pattern. This observation, subsequently, warns about generalization of binding behavior of a given B subunit, based on the binding behavior of a single related subunit of the same family.

(2) It seems that not just the specific Aα residue that is mutated determines the binding pattern to B-type subunits, but especially the **amino acid in which it is substituted**. This is demonstrated most clearly by the differences in B-type subunit binding of the R183G/Q/W mutants. For example, R183G and R183W both still interact with PR72, while R183Q is completely unable to do so.
(3) Although the mutations cluster in B subunit-binding HEAT-repeats 5 and 7, the PP2Ac interaction is decreased (except for R249H which shows WT binding to PP2Ac and B subunits). In agreement, Aα deletion mutants of HEAT-repeats 5 and 7 show reduced binding to the C subunit [23]. Together, these data point towards a complex relationship between B and C subunits within the holoenzyme in which **stable C subunit binding to Aα is dependent on the presence of the B subunit**.
(4) In contrast, B'γ, B'δ, PR72 and STRN3 can interact with a mutant Aα protein containing HEAT-repeats 1 to 10 (amino acids 1 to 410) and unable to interact with PP2Ac, to form **B'γ/B'δ/PR72/STRN3-Aα dimeric complexes**. Formerly, only proof existed for a PR72-Aα complex without PP2Ac in the format of a crystal structure [25]. For the B'δ, PR72, PR70 and the striatin [74, 75] subunits, however, it was previously shown that they are able to associate with a deletion mutant of the entire PP2Ac C-tail, already suggesting that these B-type subunits do not need stabilizing contacts with the catalytic subunit to form trimeric holoenzymes. For B'γ, the PP2Ac C-terminal determinants facilitating its incorporation in the holoenzyme were not reported before. Bα/β and B'α/β/ε subunits do require contacts with PP2Ac, in particular with the conserved C-terminal tail, to form a stable holoenzyme [74].
(5) Finally, we show for the first time **gain-of-binding of the B''' STRN family members** and of the cellular PP2A inhibitor **TIPRL1** to Aα mutant proteins.

Furthermore, the dominant mechanism-of-action, brought to light by the characterization of the ID-associated Aα mutations, was further substantiated upon ectopic expression of P179R, R182W (ID-associated), R183G, R183Q and S256F mutants in the endometrial cancer cell line HEC-1-A. This resulted in a severe phenotype for R183G/Q and S256F and a milder phenotype for P179R and R182W when evaluating AIG and tumor formation in mice. So, these mutations indeed must function via a dominant effect, rather than via haploinsufficiency. For the latter, a phenotype only emerged when WT Aα levels were first reduced by half, and was completely dependent on the loss of certain trimeric holoenzymes (particularly, PP2A-B'γ) [96]. In contrast, the cancer phenotype within our model is not dependent on the loss of binding of regulatory B subunits, but rather on the B subunits that maintained binding to mutant Aα (Figure 7). Indeed, the mutants could still form substrate-trapping complexes with these B subunits, with impaired catalytic activity and able to compete with WT Aα containing, active complexes for substrate binding. Moreover, the severity of the phenotype may be linked to the number of B subunits present in these inactive complexes, as well as by their absolute binding abilities, shown by the interaction data in HEK293 and HEC-1-A cells. In this regard, the mutant showing the strongest tumorigenic potential, R183Q, interacts the best with several B56/PR61B' family members, especially B'γ and B'δ, and binds most TIPRL1. Important to emphasize, the more B subunits are incorporated in substrate-trapping, inactive complexes, the less redundancy there will be for dephosphorylation of a given substrate and the more severe the cancer phenotype will manifest, as demonstrated by our biochemical and functional data. In support of this view,

suppression of individual B-type subunits could only partially transform HEK-TER cells and overexpression of B'γ could only partially rescue the SV40 st tumorigenic phenotype [86].

PPP2R1A mutation	Dominant-negative complexes	Gain- of -binding	Pathway hyperactivation/inhibition
P179R	B'δ, PR72	STRN3, TIPRL1	GSK3β ↑ Ras/Raf/MEK ↓
R182W (ID)	$B'\gamma_3$, B'δ, B'ε	STRN3, TIPRL1	GSK3β ↑ Ras/Raf/MEK ↓
R183G	$B'\gamma_{2/3}$, B'δ, PR72	STRN3, TIPRL1	Akt/GSK3β ↑ mTOR/p70S6K ↑ Ras/Raf/MEK ↓
R183Q	$B'\gamma_{1/2/3}$, B'δ	STRN3, TIPRL1	Akt/GSK3β ↑ mTOR/p70S6K ↑ Ras/Raf/MEK ↓
S256F	B'β, $B'\gamma_{1/2/3}$, B'δ	STRN3, TIPRL1	Akt/GSK3β ↑ mTOR/p70S6K ↑ Ras/Raf/MEK ↓

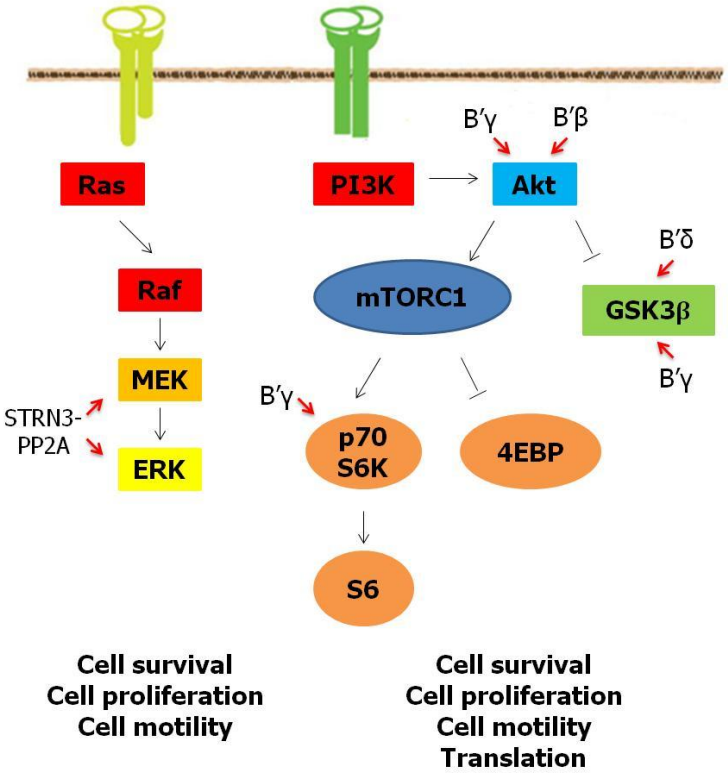

Figure 7: The upper panel shows the disease-associated *PPP2R1A* mutations, which result in the formation of dominant-negative complexes containing TIPRL1, leading to hyperactivation (↑) of components within the Akt/GSK3β and mTOR/p70S6K signaling pathways. The severity of the cancer phenotype is linked to the number of B subunits present in these inactive complexes, because the more B subunits are incorporated, the less redundancy there will be for dephosphorylation of a given substrate (lower panel). Gain-of-binding of PP2A to STRN3 might be responsible for the inhibition (↓) seen on the Ras/Raf/MEK pathway. Figure adapted from [131].

Incorporation of TIPRL1 in dominant-negative mutant Aα complexes

Our data suggest a novel pathological function of TIPRL1 via gain-of-binding to endometrial cancer- and ID-associated mutant Aα proteins, and resulting in formation of dominant-negative PP2A complexes. This was brought to light by the MS-based approach in HEC-1-A and validated by immunoblotting. Furthermore, we were able to show the existence of TIPRL1-(mutant Aα)-C complexes and proved TIPRL1 incorporation in B'δ-containing holoenzymes, resulting in a lower activity of B'δ-(mutant Aα)-C complexes. Moreover, addition of recombinant TIPRL1 to isolated B'γ/δ-(mutant Aα)-C complexes resulted in decreased C activity, compared to WT complexes. TIPRL1 is a ubiquitously expressed PP2A inhibitory protein, of which the physiological function is largely unknown, but that appears overexpressed at moderate frequency or mutated at low frequency in several human cancer types (cBioportal). The PP2A inhibitory activity resides in the C-terminal part that is lacking in the shorter *TIPRL* splice variant, TIPRL2, which does not interact with or inhibit PP2A. TIPRL1 was shown to bind PP2Ac, without the presence of WT A or any B-type subunit [179]. It has been hypothesized that TIPRL1 might play a role in PP2A biogenesis as one of the proteins that controls the activity of the newly translated PP2Ac, similar to PME1 [62]. Alternatively, it might rather play a role in PP2Ac capturing and inhibition after PP2A holoenzyme disassembly following cellular stresses, like DNA damage [62]. However, these hypotheses are highly speculative and formulated based on fragmented and even contradicting results. Our data might give support though for a role of TIPRL1 in the biogenesis of active PP2A trimers through an interaction with Aα. Although TIPRL1 biochemistry is poorly understood, it was reported that TIPRL1 has a positive effect on mTORC1 signaling through association with PP2Ac, and that TIPRL1 overexpression results in hyperphosphorylation of p70S6K and 4E-BP [180]. Notably, we also found increased p70S6K phosphorylation upon ectopic expression of mutant Aα (showing gain-of-binding to TIPRL1).

In summary, our data identify TIPRL1 gain-of-binding to B-(mutant Aα)-C substrate-trapping complexes as a likely cause of increased endometrial cancer cell growth. By extension, such increased recruitment of TIPRL1 into ID-associated mutant Aα-B'δ-C complexes may provide the mechanistic explanation for the observed decrease in specific PP2Ac activity in these complexes as well.

Expression of dominant-negative Aα mutants correlates with increased mTOR/p70S6K and Akt signaling, but with decreased ERK signaling

The hyperactivation of Akt and mTOR pathway members Akt, GSK3β, p70S6K and S6 supports a dominant-negative effect on the B56/PR61/B' family members, particularly on B'β-, B'γ- and B'δ-containing PP2A trimers (Figure 6). In Hela cells, B'γ, but not B'δ, was reported to be responsible for dephosphorylation of p70S6K, since siRNA-mediated knockdown of *PPP2R5C* (but not *PPP2R5D*) resulted in hyperphoshorylation of p70S6K [181]. Also, *Drosophila* PP2A-B', for which B'γ and B'δ are the human orthologues, interacts with and dephosphorylates p70S6K [181]. Furthermore, B'γ was identified as an important tumor suppressive B-type subunit, since its knockdown (partially) replaced SV40st in the HEK-TER

system for human cell transformation [86, 87]. B'γ knockdown was accompanied by Akt and Wnt pathway activation [87]. Several PP2A holoenzymes can interact with and dephosphorylate Akt, depending on the context [30]. However, studies in mammalian cells, *Drosophila* and *C. elegans* convincingly linked PP2A PR61/B' subunits to Akt dephosphorylation, in particular the PR61/B'β and B'γ isoforms. Mainly PR61/B'β dephosphorylates Akt in liver and was shown to be regulated by insulin/IGF signaling [30]. Additional tumor suppressive functions have been reported for B'γ. B'γ3 was shown to be enriched in the nucleus during S phase where it regulates CDK inhibitor p27KIP1 to restrain cell cycle progression [182]. B'γ 1 and 3 isoforms also promote p53 stability through Thr55 dephosphorylation during DNA damage [183]. So, multiple pieces of evidence firmly establish B'γ as a major tumor suppressive B subunit. Furthermore, unpublished data from our lab identified B'δ as a tumor suppressor *in vivo*, since *Ppp2r5d* KO mice spontaneously develop several primary tumors, with high incidence of HCC and lymphoma. In this model, lack of B'δ also results in hyperphosphorylation of GSK3β and, subsequently, in increased oncogenicity of the c-Myc oncogen. Indeed, inactivation of GSK3β results in lack of Thr58 phosphorylation in the oncogenic c-Myc form (phosphorylated on Ser62) and this leads to c-Myc stabilization [166]. In proliferative vascular smooth muscle cells, a functional redundancy has been reported between PP2A-B'γ and PP2A-B'δ towards GSK3β [184]. More controversy exists on which regulatory B subunit is responsible for dephosphorylating ERK, with some reports identifying the B56/PR61/B' family members B'β and B'γ, but not the B55/PR55/B family members [185, 186], while others disprove the role of B56/PR61/B' family members in ERK dephosphorylation and put forward Bα and Bδ as the responsible phosphatases [187]. This nicely illustrates how the action of PP2A phosphatases on a given substrate may be particularly dependent on the cellular context and the specific repertoire of PP2A B-type subunits present in a given cell or tissue.

All Aα mutants tested show gain-of-binding to the striatins (B''' subunits)

It is very well possible that the STRN3 gain-of-binding to mutant Aα might also contribute to the observed phenotype in the endometrial cancer cells. The obtained Aα interactomes from the mutant and WT HEC-1-A cells highlighted the presence of multiple STRIPAK components, including all three striatin family members (STRN, STRN3 and STRN4), Mammalian Sterile 20-like *kinase* 3 (MST3), Striatin Interacting Protein 1/2 (STRIP1/2, also FAM40A/B), STRIP2-interactor MYH9, sarcolemmal membrane–associated protein (SLMAP), cerebral cavernous malformation 3 (CCM3), phocein (Mob4, also PREI3), suppressor of IKKε (inhibitor of NF-κB kinase ε) (SIKE), fibroblast growth factor receptor 1-oncogenic partner 2 (FGFR1OP2) and Cortactin Binding Protein 2 N-Terminal Like (CTTNBP2NL). STRIPAK and STRIPAK-like complexes are multiprotein signaling complexes involved in the regulation of the cell cycle, cell growth and transformation, cell polarity, apoptosis, cell survival, Golgi assembly, and cell migration [50]. Consistent with this wide range of functions, over 100 different variants of STRIPAK complexes have been predicted from different combinations of STRIPAK components. To date, PP2A A and C subunits are the only striatin family-associated proteins found in nearly all identified complexes [50]. Striatins form homodimers that interact with A and C as a 2:2:2 heterohexamer, and homodimerization is essential for interaction with PP2A

subunits [188]. However, the functions of PP2A within the different STRIPAK complexes are only beginning to emerge:

(1) Very recently, a paper reports the function of a STRIPAK complex containing multiple members identified in our (mutant) Aα interactomes. PP2A-STRN associates with germinal center III kinases (GCKIII) MST3, MST4 and Serine/Threonine Kinase 25 (STK25) through the adaptor protein CCM3, and PP2A is believed to function as a negative regulator of the associated kinases. MST3 promotes apoptosis in Jurkat cells via its effects on NF-κB signaling. PP2A inhibits the **MST3 kinase** via dephosphorylation [189] and, subsequently, counteracts MST3-mediated apoptosis pointing towards a tumor promoting function of PP2A-STRN within certain STRIPAK complexes [188]. Of note, CCM3 is mutated in familial cerebral cavernous malformation, a condition of abnormal brain capillary architecture, associated with symptoms ranging from dizziness and headaches to severe stroke and death [190].

(2) In addition, positive regulation of STRIPAK on **ERK signaling** was suggested in Drosophila and in mammalian cells, since a STRIPAK (dSTRIPAK) complex containing dGckIII and PP2A stimulates the ERK pathway and since mammalian GCKIII kinases, MST3 and MST4, are important for maintaining basal ERK phosphorylation [191-193]. Interestingly, mutant Aα HEC-1-A cells show reduced phosphorylation of ERK, consistent with a disturbed phosphorylation/dephosphorylation balance within STRIPAK due to the enhanced STRN3-PP2A association (Figure 7). Moreover, this suggests a gain-of-function of STRN-PP2A, rather than a dominant-negative effect on STRIPAK substrates. Absence of TIPRL1 incorporation in these complexes might be explained by the different structure/composition of the multiprotein A-C-STRN signaling complex compared to A-C-B/B'/B" heterotrimers.

(3) STRIPAK complexes are also involved in the regulation of **Hippo signaling**. SLMAP is responsible for recruiting MST1/2 kinases to the STRIPAK complex [51]. When MST1/2 kinases are phosphoylated, they phosphorylate and activate large tumor suppressor kinase 1 and 2 (LATS1/2), which in turn phosphorylate the transcriptional coactivators Yes-associated protein 1 (YAP1) and Tafazzin (TAZ) leading to their cytosolic retention or degradation. In contrast, absence of phosphorylation causes YAP1 and TAZ to translocate into the nucleus, ultimately inhibiting apoptosis and stimulating proliferation. Moreover, siRNA-mediated knockdown of PP2Ac results in an increased abundance of phoshorylated **MST1/2** [51], leading to the suggestion that gain-of-binding of PP2A mutant Aα and C subunits to this STRIPAK complex might indeed block apoptosis and stimulate cell proliferation by activating Hippo signaling.

(4) Finally, STRIPAK complexes play a major role in cancer cell **migration and invasion**, which may be highly relevant for tumor metastasis. Striatin depletion alters the localization of the tight junction protein ZO-1 and affects the organization of F-actin, suggesting a contribution of Striatin in cell-cell adhesion [194]. Another STRIPAK complex, containing STRN3, PP2A, CMM3, STRIP1 (FAM40A) and STRIP2 (FAM40B), regulates the mode of cancer cell migration by facilitating the interaction of the actomyosin network with either the plasma membrane or with the extra-cellular matrix, and this through control of **MST3/4-kinase** activity, with high and low activity respectively. Interaction with the plasma

membrane facilitates 3D migration, while interaction with the extra-cellular matrix favours 2D migration [56]. Dedicated functional assays in HEC-1-A cells expressing the cancer-associated Aα mutants should provide further insights into whether cell adhesion, migration of invasion into the extracellular matrix are affected, and potentially, increased, as opposed to WT Aα expressing cells. This could possibly lead to the conclusion that Aα mutation might also contribute to the metastatic potential of the endometrial cancer cells.

Potential contribution of other Aα interactome constituents to the cancer growth phenotype

Of course, additional PP2A interaction partners, including those amongst the putatively novel interactors, might also contribute to the observed cancer phenotype. Some of the established Aα partners, CCDC6, ANKLE2, TMPO, PP4, liprin α and the Integrator complex, have already been reported to have links with cancer pathology.

CCDC6 was originally identified as a translocation partner with RET in human thyroid papillary carcinomas generating the RET/PTC1 oncogene [195]. This rearrangement was also detected in lung adenocarcinomas and *CCDC6* gene rearrangements with other genes have been reported in solid tumors and leukemia. Mechanistically, CCDC6 is a substrate of ATM and aids to protect genome integrity by negatively modulating PP4c activity directed towards H2AX after DNA damage. Furthermore, CCDC6 interacts with CREB1 to repress its transcriptional activity [195]. Interaction between CCDC6 and Aα mutants was variable; some displayed increased binding compared to WT Aα (P179R, S256F), others showed decreased binding (R182W and R183G/Q).

PP2A regulates barrier-to-autointegration factor (BAF) chromatin recruitment during mitotic exit and is required for BAF's function in nuclear envelope assembly [196]. Both *C. elegans* and human PP2A complexes are required to dephosphorylate BAF during mitotic exit and thus allow its efficient recruitment to chromatin. ANKLE2 serves a dual role in this process by (1) supporting PP2A-mediated BAF dephosphorylation and (2) by inhibiting the mitotic BAF kinase, VRK-1 [196]. ANKLE2 is a member of the LAP2-Emerin-MAN1 (LEM) domain protein family, as well as TMPO (or LAP2α). TMPO, present in the nucleoplasm, has been reported to control cell proliferation by affecting the activity of retinoblastoma protein in tissue progenitor cells and numerous studies showed upregulation of LAP2α in cancer. Both LEM proteins show variable binding to mutant Aα in comparison with WT Aα [197].

PP4 is believed to be tumor-promoting, since overexpression of PP4C-PP4R2-PP4R3α was discovered in human primary breast and lung tumors, and subsequent inhibition of PP4C expression sensitized these cells to cisplatin treatment [198]. Also in pancreatic ductal adenocarcinoma PP4C is overexpressed, and this is associated with poor prognosis in patients with stage II disease [199]. More recently, increased PME-1 expression and increased PME-1 binding to PP4 was also found in endometrial cancers, although the contribution of potential PP4 dysregulation in PME-1 overexpressing tumors remains unclear [140]. Noteworthy, TIPRL1 is also a reported interactor of PP4 [114] and previously reported Aα-containing PP4 complexes have been shown to specifically harbor B'δ and B55α/δ

subunits [200, 201]. Thus, it is definitely worthwhile to further investigate the potential role of mutant Aα -PP4 complexes in the cell biology of endometrial cancer.

The B'γ3 and B'δ binding partner PPFIA1 (Liprin α1) was found to be required for metastasis and invasion of the highly invasive breast cancer cell line MDA-MB-231 [202, 203] and is overexpressed with high frequency in several tumor types (cBioportal), suggesting it is an oncogen. Our MS data show decreased binding of liprin α1 to all Aα mutants tested. However, since the we do not know how PP2A affects Liprin α1 within the complex, it is hard to speculate about the contribution of Liprin α1 loss-of-binding to the cancer phenotype.

Finally, the Integrator complex functions in gene expression regulation [204]. It is a U-rich small nuclear RNAs (UsnRNAs) 3'-end processing factor via its association with RNA polymerase II. UsnRNAs represent a family of small non-coding RNAs, essential for the biosynthesis of ribosomal RNA, removal of introns and proper expression of histone mRNA, and are highly expressed throughout development and the cell cycle of all cells. Again, the role of PP2A within the Integrator complex is completely unknown, as well as a potential link to cancer.

Contribution of STRN3 gain-of-function to the ID phenotype?

Despite the common defect in B'δ-dependent dephoshorylation in the 16 reported ID cases here, the Aα cases were more severely affected than the B'δ cases and show some differential symptoms; all had severe ID, absent speech, small head size and partial or complete corpus callosum agenesis. This difference is in line with the expected greater difficulty to compensate for a general scaffolding (A) subunit dysfunction than a specific regulatory (B) subunit dysfunction. However, the data obtained from the endometrial cancer-associated Aα mutations might suggest the contribution of additional factors to the phenotype, possibly the gain-of-function of **STRIPAK complexes**. The striatin family members are highly expressed in brain, especially in the striatum, hence their name. They concentrate in dendritic spines, the sites of most of the excitatory synapses, and are involved in the control of motor function, since downregulation of striatins is associated with reduced motor activity [205]. Downregulation also results in reduced growth of dendrites [205]. So, the striatin family is essential for dendritic spine formation, possibly in a Ca^{2+}-regulated manner, since Ca^{2+} is required for proper neuronal maturation [206]. Dendritic spine localization is dependent on CTTNBP2, a CTTNBP2NL-holomogue, of which the knock down also causes a reduction in the number of dendritic spines [57]. Interestingly, CTTNBP2 is being investigated as a possible autism susceptibility gene, because a *de novo* 2-base-pair frame shift deletion in CTTNBP2, likely disturbing protein function, was found in a male autism patient [207]. Furthermore, striatin's interaction with phocein (Mob4) might be involved in synaptic plasticity via spine remodeling by endocytosis [208]. Striatins associate with estrogen receptor α (ERα) and are involved in the non-genomic actions of estrogen [209]. In brain, loss of estrogen is associated with vulnerability to epilepsy, memory problems, defects in fine motor coordination and reaction time, and depression and anxiety [209]. Non-genomic functions of estrogen include rapid action on excitability of neuronal and pituitary cells, activation of MAPK and Akt pathways, protection of neurons from excitotoxin-

and free radical-mediated damage, and regulation of calcium channels and calcium ion entry. Estrogen is also involved in formation of excitatory synapses in the hippocampus [209]. Furthermore, striatin co-localizes with APC in PC12 cells during neurite outgrown following NGF treatment [194].

Therefore, the literature highlights the indisputably important function of striatins in brain biology and may predict a pathological role for gain-of-function STRN-(mutant Aα)-C complexes in ID.

All ID-associated *PPP2R1A* mutations are common to uterine cancer

With advances in genome sequencing, several hundreds of new candidate genes for neurodevelopmental disorders have been identified and need to be prioritized. Among the factors that determine whether a gene is a "disease gene" worthwhile to pursue, are (1) recurrence of the *de novo* mutation, (2) the position of the mutation in the protein (clustering), (3) presence of the mutant protein within a disease-implicated protein network and (4) **presence of a link with cancer biology** [165]. Indeed, many recurring genes and pathways have been implicated in both neurodevelopmental diseases and cancer, e.g. mutation of the tumor suppressor gene *PTEN* found in Cowden syndrome. Strikingly, in some cases identical point mutations are causative for cancer when mutated somatically and for neurodevelopmental disorder when mutated in the germline. This is indeed the case for **all the ID-associated Aα mutations** P179L, R182W and R258H (but not for the B'δ mutations). Also for MCAP/MPPH, identical mutations within the PI3K/Akt pathway have been found in cancer. For these patients, a mild increase of 3% in cancer incidence was reported with detection of Wilms tumor, leukemia, medulloblastoma and meningioma in patients aging from 10 months to 18 years [210]. However, none of our patients (ages 1 to 11 years old) have been diagnosed with or treated for cancer yet. In addition, the single ID-associated mutation tested in our cancer model (R182W) seems to have a very mild tumorigenic potential in endometrial cancer, with no hyperactivation of oncogenic Akt and mTOR signaling pathways compared to R183G/Q and S256F, and no significant difference in AIG and *in vivo* tumor formation in mice compared to WT Aα. Only further patients and patient follow-ups will provide a clear answer, but a major cancer risk seems unlikely. Finally, it could be hypothesized that the timing of the mutational event determines the different outcomes (germline/constitutively vs somatic), as well as the specific genetic background and the cellular context [165]. In case of PP2A, the latter might be very important since B'δ and STRN3 are highly enriched in the brain, potentially explaining why the germline mutations found in the ID patients seem to majorly affect this specific organ only.

Therapeutic implications for the treatment of *PPP2R1A* mutant endometrial cancers

Compared to Type I endometrioid carcinoma, serous uterine carcinoma (type II) is less common but is characterized by high mortality due to a tendency for early metastasis and resistance to conventional chemotherapy. Besides *TP53* mutations, occurring in up to 80-

95% of cases, relatively few additional molecular genetic aberrations were reported in this cancer type, among which *PPP2R1A* appears the most frequently altered in 18.4 up to 43.2% of cases, depending on the study [100-103, 105, 133-135]. Also, *PPP2R1A* mutations occur in high-grade endometrioid cancer and is therefore associated with poor outcome and aggressive behavior of both the endometrioid and serous types of endometrial adencarcinoma. This opens interesting perspectives for the use of *PPP2R1A* as a therapeutic biomarker.

Our functional data would indeed suggest that *PPP2R1A* mutant tumor cells could be targeted by **kinase inhibitors directed against the Akt and mTOR signaling pathways**, like Akt, mTORC1, isoform-specific PI3K, Pan-PI3K, dual PI3K/mTOR and mTORC1/2 inhibitors, or a combination of these drugs [130]. Moreover, since we do not observe oncogenic activation of the Ras/Raf/MEK pathway (probably due to STRN-PP2A gain-of-function), cross-talk between this pathway and the Akt/mTOR signaling pathways, often the basis for resistance, might not be a big issue in mutant Aα endometrial cancer.

Combined, our observations predict a good therapeutic efficiency for Akt/mTOR inhibitors and might suggest a substantial benefit of stratification of patients based on mutant or WT Aα status of the tumor. In addition, the PAD **PPZ** might have therapeutic potential to reactivate mutant Aα. PPZ is believed to bind to the A subunit [162]. One plausible explanation for its reactivating ability is that PPZ might enhance the local aspecific activity of PP2A holoenzymes by dissociating the B subunit, therefore enhancing the activity of the "uncontrolled" A-C dimer. If this is true, then PPZ might just as well dissociate remaining mutant Aα-B' subunit interactions, and alleviate the dominant-negative effects of these complexes.
Finally, our data highlight the therapeutic potential of inhibiting TIPRL1. Therefore, it is of great importance to gain more insight into the PP2A inhibitory function of TIPRL1 and the TIPRL1-PP2A(Aα) interaction to ultimately be able to design or screen for small molecules targeting this PP2A-inhibitor interaction. Alternatively, since PPZ and TIPRL1 both seem to bind the A subunit, there is a possibility that PPZ might dissociate TIPRL1 from PP2A as another mechanism-of-action.

8 Future perspectives

FUTURE PERSPECTIVES

Regarding the role of PP2A dysfunction in ID:

Future experiments should focus on:

(1) the identification of neuronal substrates of B'δ, whose hyperphosphorylation contributes to the disease. To this end, we could exploit potential differential binding of relevant substrates to WT B'δ, to the E198K mutant (substrate trapping) and to P53S (mutant with potential loss of substrate binding)
(2) the generation and characterization of appropriate cellular and *in vivo* models of B56deltopathy. Essentially, these should be knock-in (KI) models, technically achievable for instance by using CRISPR-Cas9 technology. This technology allows to introduce the relevant point mutation into the *PPP2R5D* or *PPP2R1A* locus. Such B56deltopathy models should be instrumental to further investigate or validate the consequences of PP2A dysfunction on neuronal signaling and brain function. Moreover, they could be utilized to test therapeutics (e.g. kinase inhibitors or PADs) pre-clinically, and to monitor for potential increased susceptibility to cancer development.
(3) In the meantime, the lab became aware of additional genetic alterations in PP2A genes in brain diseased patients. Therefore, we should continue to collaborate with clinical geneticists to provide biochemical and functional evaluation of newly discovered ID-associated mutations in PP2A subunit genes. In this respect, I recently already obtained preliminary biochemical data on the reported B'γ T157del mutation in an overgrowth patient [152], finding significant binding defects with A and C subunits, and hyperphosphorylation of Akt Thr308 upon its ectopic expression in HEK293 cells.

Regarding the biomarker role of *PPP2R1A* mutation in endometrial cancer:

Our data provide a molecular basis for improved design of targeted therapies, which should be further experimentally tested. Future experiments should focus on:

(1) targeting the affected Akt/mTOR signaling pathways, testing multiple drugs and their combinations, like Akt, mTORC1, isoform-specific PI3K, Pan-PI3K, dual PI3K/mTOR and mTORC1/2 inhibitors. To this end, their therapeutic effects in Aα mutant HEC-1-A cells, e.g. on cell survival, oncogenic signaling, AIG and tumor formation in mice, should be tested.
(2) evaluating PPZ as a potential PP2A (re)activator of mutant Aα in the same experimental set-up as described above.
(3) gaining more insight into the relationship between TIPRL1 and PP2A(Aα) to ultimately be able to disrupt their interaction, perhaps via PPZ.
(4) studying more elaborately the contribution of the PP2A-STRN gain-of-function to the observed cancer phenotype by evaluating the phosphorylation states of STRIPAK-associated kinases (e.g. MST3) and substrates, and also by determining potential changes in cell adhesion, migration and invasion of the mutant Aα expressing cells.

9 Summary

SUMMARY

Here, we report inherited dysregulation of protein phosphatase activity as a novel cause of intellectual disability (ID). *De novo* missense mutations in 2 PP2A subunit genes (*PPP2R1A* and *PPP2R5D*) were identified in 16 individuals with ID of unknown etiology. Mutant B'δ was A and C binding–deficient, while mutant Aα subunits bound B'δ well but were unable to bind C or bound a catalytically impaired C, suggesting a dominant-negative effect where mutant subunits hinder dephosphorylation of B'δ-anchored substrates. The same dominant-negative mechanism caused increased cancer cell growth in high grade uterine cancers bearing somatic *PPP2R1A* missense mutations, some of which were identical to all the Aα mutations found in ID. *PPP2R1A* mutation represents one of only a few genomic alterations occurring with high frequency in serous endometrial carcinoma and carcinosarcoma, typically aggressive uterine cancers. This thesis reports on the biochemical and functional consequences of eleven recurrent mutations clustering into Aα HEAT-repeats 5 and 7. Besides the predicted loss-of-function effects on formation of some PP2A holoenzymes, we confirm the dominant-negative effects of these mutations in the cancer context and this on specific, tumor suppressive PP2A complexes. Dominant-negative Aα mutants retain binding to specific B-type subunits, but form substrate-trapping complexes with impaired phosphatase activity due to increased incorporation of a cellular PP2A inhibitor, TIPRL1. In addition, we reveal gain-of-binding of the Aα mutants to the B'''/striatin subunits of PP2A, which we suspect also contributes to the dominant behavior of the mutants. Accordingly, ectopic expression of these mutants in endometrial carcinoma cells increases anchorage-independent growth and tumor formation *in vivo*, and causes hyperphosphorylation of several oncogenic PP2A substrates in the Akt and mTOR signaling pathways. Although identical Aα mutations are found in both pathologies, a major cancer risk for the ID patients seems unlikely. Together, our data not only reveal how mutations affect PP2A assembly and function, oncogenic signaling and ID disease pathogenesis, they also provide a basis for improved design of targeted therapies.

SAMENVATTING

Wij rapporteren hier een aangeboren afwijking van proteïne fosfatase activiteit als een nieuwe oorzaak van mentale retardatie (MR). *De novo* missense mutaties in 2 PP2A subeenheid genen (*PPP2R1A* en *PPP2R5D*) werden geïdentificeerd in 16 personen met MR met een ongekende oorzaak. Mutante B'δ regulatorische subeenheden waren deficiënt in A en C subeenheid binding, terwijl mutante Aα subeenheden B'δ nog goed bonden maar verstoorde katalytische activiteit vertoonden. Deze data wijzen op een dominant-negatief effect waarbij de mutante subeenheden niet meer in staat zijn om B'δ substraten te defosforyleren. Hetzelfde dominant-negatieve mechanisme veroorzaakt verhoogde agressiviteit/groei in baarmoederkankercellen met somatische *PPP2R1A* missense mutaties, waarvan sommigen identiek zijn aan alle Aα mutaties gevonden bij MR patiënten. *PPP2R1A* mutatie vertegenwoordigt één van de weinige genomische veranderingen die zich voordoen met hoge frequentie in sereuze baarmoederkanker en in carcinosarcoom, beide agressieve vormen van uteriene kanker. Deze thesis toont de biochemische en functionele gevolgen van elf baarmoederkanker-geassocieerde en recurrente mutaties in *PPP2R1A* die samenklusteren in HEAT-repeats 5 en 7. Naast het voorspelde verlies van interactie met sommige PP2A subeenheden, bevestigen wij de dominant-negatieve effecten van deze mutaties in de kanker context en dit op specifieke gekende tumor onderdrukkende PP2A complexen. Dominant-negatieve Aα mutanten behouden binding aan specifieke B regulatorische subeenheden, maar vormen substraat-"trapping" complexen met verminderde fosfatase activiteit te wijten aan verhoogde incorporatie van een cellulaire PP2A inhibitor, TIPRL1. Zo toonden we aan dat ectopische expressie van deze mutanten in baarmoederkankercellen resulteerde in een sterker tumor fenotype *in vitro,* en *in vivo* in muizen. Verder kon verhoogde fosforylatie van verschillende oncogene eiwitten binnen de Akt en mTOR signaaltransductiewegen hiermee gecorreleerd worden. Tot slot, toonden we voor het eerst ook een verhoogde binding van de Aα mutanten aan de B'''/striatine subeenheden van PP2A aan, waarvan we vermoeden dat deze eigenschap eveneens bijdraagt aan het dominante karakter van deze mutanten. Alhoewel identieke Aα mutaties zijn teruggevonden in beide ziektebeelden, lijkt een groot kankerrisico voor de MR patiënten onwaarschijnlijk. Samengevat, tonen onze data niet enkel hoe specifieke, ziekte-geassocieerde mutaties invloed hebben op PP2A vorming en functie, oncogene signalering en MR pathogenese, zij vormen ook een rationele basis voor de ontwikkeling van verbeterde doelgerichte therapieën.

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11 Epilogue

EPILOGUE

CURRICULUM VITAE

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PROFESSIONAL CAREER

Publications

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Awards

Isabelle Oberlé Award for Research on Genetics of Mental Retardation. The EUROPEAN HUMAN GENETICS CONFERENCE 2015. Glasgow, UK, 6-9 June 2015.